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Honey Authenticity: Development and validation of a UPLC-ESI-QToF MS method for the determination of phenolic compounds

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Αυθεντικότητα μελιού: Ανάπτυξη και επικύρωση UPLC-ESI-QToF MS μεθόδου για τον προσδιορισμό των φαινολικών συστατικών

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ABSTRACT

Food authenticity is a rapidly growing field mostly due to public awareness concerning food quality and safety. Of special concern is the declaration of specific quality attributes in high-value products, like honey. In this way, new analytical methods are needed to be developed in order to verify the botanical or geographical origin of honey.

In this thesis, an overview on honey is presented. Features, like chemical composition, organoleptic and physicochemical characteristics and quality criteria are discussed. A special focus is made on phenolic compounds of honey. Phenolic compounds are a wide group of compounds with essential importance for honey authenticity studies. Additionally, the state of the art on food authenticity studies is comprehensively reviewed. A scientometric evaluation of the field is composed, highlighting the research trends and giving insight on emerging approaches of this evolving field. Techniques used in honey authenticity studies alongside with authenticity markers are cited.

The development and validation of UPLC-ESI-QToF MS method for the determination of phenolic compounds in honey is presented in the experimental part. It is proved that the developed validated method was capable for the determination of phenolic compounds in honey. Thereafter, 10 different unifloral, conventionally and organically produced honeys from Poland and Greece were investigated for their phenolic content. The concentration of the determined polyphenols ranged from 0.038 to 5.0 mg/Kg. The unifloral honeys with the greater phenolic content were buckwheat and fir honey, while acacia and arbutus honey contained low amount of these compounds. Moreover, fir honey featured a high quercetin concentration compared to the other matrices. Similarly, heather honey had a high amount of cinnamic acid, while acacia honey was measured with an increased concentration of ferulic acid. Consequently, all the previously mentioned compounds may be used as potential authenticity markers. Furthermore, the production type of honey was also investigated in order to find if organic production affects phenolic

compounds content. Finally, suspect screening workflow was performed on oak honey samples and three flavonoids were identified and semi-quantified.

In conclusion, the results obtained in the present work illustrate the importance of investigating polyphenols content in honey authenticity studies.

SUBJECT AREA: Food Analysis; Food Authenticity

KEYWORDS: honey authenticity, phenolic compounds, botanical origin, geographical origin, RPLC-QToF-MS, potential authenticity markers

ΠΕΡΙΛΗΨΗ

Η αυθεντικότητα τροφίμων είναι ένα ραγδαία αναπτυσσόμενο γνωστικό αντικείμενο, εξαιτίας του αυξημένου ενδιαφέροντος που δείχνει η κοινή γνώμη σε θέματα ασφάλειας και ποιότητας τροφίμων. Η διασφάλιση των χαρακτηριστικών ποιότητας σε τρόφιμα υψηλής εμπορικής αξίας, όπως το μέλι, είναι ιδαίτερης σημαντικότητας. Έτσι, νέες αναλυτικές τεχνικές αναπτύσσονται με σκοπό να ταυτοποιήσουν την γεωγραφική ή βοτανική προέλευση του μελιού.

Αρχικά, στην παρούσα διατριβή παρουσιάζεται μια επισκόπιση σχετικά με το μέλι. Η χημική σύσταση, τα οργανοληπτικά και φυσικοχημικά χαρακτηριστικά καθώς και τα κριτήρια ποιότητας παρατίθενται. Ειδική αναφορά γίνεται στα φαινολικά συστατικά του μελιού. Οι φαινολικές ενώσεις είναι μια πολυάριθμη ομάδα συστατικών με καθοριστική σημασία για την αυθεντικότητα του μελιού. Επιπλέον, γίνεται ανασκόπηση των τελευταίων εξελίξεων στις μελέτες αυθεντικότητας τροφίμων. Οι ερευνητικές τάσεις του τομέα παρουσιάζονται, υπογραμμίζοντας τις προοπτικές των αναλυτικών τεχνικών στην αυθεντικότητα μελιού μαζί με δείκτες αυθεντικότητας που έχουν αναφερθεί στη βιβλιογραφία.

Στο πειραματικό μέρος παρουσιάζεται η ανάπτυξη και επικύρωση της UPLC-ESI-QToF MS μεθόδου για τον προσδιορισμό των φαινολικών συστατικών στο μέλι. Αποδεικνύεται ότι η μέθοδος που αναπτύχθηκε είναι κατάλληλη για τον προσδιορισμό των φαινολικών συστατικών στο μέλι. Στη συνέχεια, έγινε έλεγχος του περιεχομένου σε φαινολικά συστατικά σε 51 δειγματα από 10 διαφορετικά μονοποικιλιακά μέλια, που έχουν παραχθεί τόσο συμβατικά όσο και βιολογικά, από την Πολωνία και την Ελλάδα. Η συγκέντρωση των αναλυτών κυμάνθηκε από 0,038 έως 5,0 mg/Kg. Τα μονοποικιλιακά μέλια που εμφάνισαν την υψηλότερη συγκέντρωση φαινολικών συστατικών ήταν από μαυροσίταρο και έλατο, ενώ αυτά με τη χαμηλότερη ήταν η ακακία και κουμαριά. Επιπλέον, το μέλι ελάτης περιείχε υψηλή συγκέντρωση κερκετίνης σε σχέση με τις άλλες μήτρες μελιού. Παρομοίως, το μέλι από ρείκι διέθετε μεγάλη ποσότητα κινναμικού οξέος, ενώ το μέλι ακακίας φερουλικό οξύ. Συνεπώς, αυτά τα φαινολικά συστατικά μπορούν πιθανώς να χρησιμοποιηθούν ως δυνητικοί δείκτες αυθεντικότητας. Συμπληρωματικά, ελέχθηκε αν ο τρόπος παραγωγής

επηρεάζει το φαινολικό περιεχόμενο του μελιού. Τέλος, έγινε σάρωση για ύποπτες ενώσεις που έχουν αναφερθεί στο μέλι σε δείγματα μελιού βελανιδιάς με αποτέλεσμα την ταυτοποίηση και τον ημιποσοτικό προσδιορισμό τριών επιπλέον φλαβονοειδών.

Συμπερασματικά, τα αποτελέσματα της παρούσας διπλωματικής μεταπτυχιακής εργασίας αντανακλούν την σπουδαιότητα του φαινολικού περιεχομένου στις μελέτες αυθεντικότητας για το μέλι.

ΘΕΜΑΤΙΚΗ ΠΕΡΙΟΧΗ: Ανάλυση και Αυθεντικότητα Τροφίμων

ΛΕΞΕΙΣ ΚΛΕΙΔΙΑ: αυθεντικότητα μελιού, φαινολικά συστατικά, βοτανική προέλευση, γεωγραφική προέλευση, RPLC-QToF-MS, πιθανοί δείκτες αυθεντικότητας

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PREFACE

This master thesis was conceived and performed at the Laboratory of Analytical Chemistry (Department of Chemistry, National and Kapodistrian University of Athens, Greece) under the supervision of the Associate Professor, Nikolaos Thomaidis.

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CHAPTER 1

Honey: An overview

1.1 Definition

Honey is a supersaturated sugar solution and is considered as a natural sweetener. Except sugars, honey is also composed of enzymes, organic acids, amino acids, carotenoids, minerals, vitamins, and aromatic substances. Additionally, honey has a great amount of phenolic compounds such as flavonoids and phenolic acids. According to the Council Directive 2001/110/EC honey is the natural sweet substance produced by Apis mellifera bees from the nectar of plants or from secretions of living parts of plants or excretions of plant-sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in honeycombs to ripen and mature [1]. The main factors that affect composition, color, flavor and aroma are the botanical and the geographical origin of honey alongside with the climate conditions and honeybee species involved in its production. Factors, also affecting the quality of honey, are weather conditions, processing, manipulation, packaging and storage time.

1.2 A flashback

The honeybee was appeared 65 million years ago, prior to the existence of human beings. Honey was the only natural sweetener until the 16th century [2]. Honeybee and honey were cited in Greek myths. A typical example was the story of the Greek God Apollon who ate nectar and ambrosia, both of them were constituted of honey, in order to be handsome. For this reason his father Zeus called him the God of Light.

Besides mythology, there is also written evidence that humans were interested in beekeeping. The first reference, dating back to 2100–2000 BC, mentions honey's use as a drug and an ointment [3]. Egyptians, Assyrians, Babylonians, Sumerians and Hittites have been used honey for both nutritional and medical purposes [3, 4]. Aristoteles (384-322 BC) has recorded that ancient Greeks were very capable beekeepers. In fact, Aristoteles was the first author who

described the behavior of the honeybees. In this way, he mentioned the existence of three different types of bees in detail the “worker bees”, the “drone bees” and the “hegemony” meaning the queen bee. In the following years the frequent use of honey was considered as a feature of well-living and longevity. Hippocrates, the father of modern Medicine, has noticed that honey helps the skin to look brighter while Pythagoras has mentioned that the consumption of honey is connected to increased energy.

The production and use of honey was increased during the Roman times. Thus, the utilization of the good was generalized both in a nutritional and a medical manner. This fact was depicted in an early encyclopedia (called The Natural History, Latin: Naturalis Historia) by Pliny the Elder (23-79 AD) which includes recipes with honey and other uses. Subsequently, Apicius a collection of Roman cookery recipes, compiled in the late 4th or early 5th century AD, highlights the importance of honey in the Roman nutrition.

In the Middle Ages, beekeeping has evolved to an occupation because of the great demand for honey and wax. Moreover, the flourish of the field was a result of the increased population and the use of wax for religious and daily needs. The Alchemists, who played a significant role in early modern science, have shown a preference for honey as a constituent in their alchemist materials. Christians were also in the same way, using both the wax in their religious practice and also in many parallelisms likening honey’s sweetness to the word of the God.

Beekeeping was affected from two conflicted factors in the Modern Times. Firstly, the “invention” of sugar has dramatically decreased the demand for honey. The consumption of sugar was globally established due to the easiness in transfer comparing to honey. An ultimate dominance of sugar was presented for the preparation of medical formulations. As a consequence, a decrease in honey production was illustrated and the beekeepers have retained the exclusive market of wax. The second element that differentiates the status in beekeeping, was the construction of the modern hives. Until then, the beekeepers were forced to destroy the hives in order to extract honey. Thus, honeybees have to construct a new hive which affects their productivity as a great amount of nectar was needed. A "Langstroth hive" is the hive that was designed by Rev. Langstroth in 1852. The advantage of this hive is that the

bees build honeycomb into frames, which can be moved with ease [5]. The frames are designed to prevent bees from attaching honeycombs where they would either connect adjacent frames, or connect frames to the walls of the hive. The movable frames allow the beekeeper to manage the bees in a way which was formerly impossible. Furthermore, honey was centrifuged from the hives which then can be reused. Indeed, the innovative honey hives bring a revolutionary era for beekeeping and improve the productivity and quality of honey.

1.3 Types of honey

The Council Directive 2001/101/EC defines that there are two types of honey according to the origin. The first type of honey obtained from the nectar of the plants and is called blossom honey or nectar honey. The amount of nectar that is collected by the honeybees is pertinent to either internal factors such as the nature of the plant or external factors like temperature, humidity, soil conditions, the amount of wind, day length [6]. The second type is the honeydew honey which is originated mainly from excretions of plant sucking insects, primarily from the order Hemiptera, on the living part of plants or secretions of living parts of plants. The production of honeydew honey is greatly depended from the plant species of a region, the climate and the local weather. However, if floral nectar is plentiful all year long, honeydew collection will be inadequate. Trees, like beech, chestnut, elm, fir, oak, pine, poplar, spruce, and willow, are the main producers of the sap that is necessary for the feeding of the insects producing the honeydew. Consumers have shown a different preference between these two types of honey. Indeed, in many European countries there is an increasing demand for honeydew honey [7]. Consequently, the differentiation of these two types of honey is of great importance in order to assure the quality of the product. The simplest way to verify the type of origin is the determination of the electric conductivity. Nevertheless, there are many differences in the composition of blossom and honeydew honey [7]. According to the Council Directive 2001/101/EC blossom honeys have electric conductivity values below 0.80 mS/cm, while the honeydew honeys exceed this value. However, there are many exceptions to this rule [8]. For this reason, a further investigation of

other characteristic like carbohydrate and phenolic compounds composition should be performed to confirm accurately the origin of the honey.

In this thesis, 9 different unifloral honeys will be studied. Particularly, honeys derived from acacia, arbutus, buckwheat, chestnut, fir, heather, linden, rape and thyme were analysed. The samples originate from Greece and Poland and were collected either directly from beekeepers or purchased from the market. Each one of these honey matrices features exceptional characteristics [9] which are comprehensively discussed in the following bullets.

- *Acacia honey:* Acacia honey is quite pale and transparent. It is important that it slowly granulates, about 1-2 years. It has the lowest glycemic index among the different types of honey – about 30 to 33.
- *Arbutus honey:* Arbutus honey is widely produced in Sardinia. It is highly sought after as it represents the last production of the bees before the winter period. Combining this fact with the superior quality of arbutus honey its high price is a rational result.
- *Buckwheat honey:* Buckwheat honey has a deep, dark brown color, pungent, earthy flavor and is high in mineral content and antioxidant compounds.
- *Chestnut honey:* Chestnut tree is one of the best sources of nectar and pollen while it can also provide honeydew, following the attack by some insects. Consequently, it can be produced both from nectar and pollen and honeydew. Additionally, the high fructose amount prevent this unifloral honey from quick granulation.
- *Fir honey:* It is originated by the honeydew that is produced by microorganisms on the wood of the fir tree. Furthermore, fir honey remains for years in liquid state due to the low amount of glucose (<30%). The extraction of honey from the combs is a destructive procedure and for this reason its production is expensive. Greece has registered since 1996 a unifloral fir honey as P.D.O (Protected Designation of Origin) under the name “ Meli Elatis Menalou Vanilia”.
- *Heather honey:* A unique physical phenomenon, called thixotropy, is observed in heather honey. Thus, the presence of colloidal proteins

results to a jelly consistence. Moreover, it shows high values of electrical conductivity, color, water and acidity. It has to be highlighted that the polish honey under the name "*Miód wrzosowy z Borów Dolnośląskich*" is a unifloral heather honey registered as a Protected Geographical Indication (PGI) product since 2008.

- *Linden honey:* Linden or lime honey has very strong and typical flavor. Electrical conductivity values are quite high, for a nectar honey, and the European Directive includes this honey in a group whose electrical conductivity may go beyond the 0.8 mS/cm limit.
- *Rape honey:* This honey is characterized by quick granulation, due to the high glucose content. As a consequence, it is regularly used as a "crystallization starter", added to other honeys to obtain a finer granulation.
- *Thyme honey:* It is mainly produced in Greece and Italy. Unifloral thyme honey presents quite high values of diastase, high values of proline, fructose and acidity

1.4 Chemical composition

The unique properties of honey are a result of its chemical composition. Hence, the knowledge of the occurrence and the quantity of any constituent is of great importance. The existence of more than 200 different components has been reported in honey [10]. Thus, compounds of honey are separated in two groups according to their abundance. The first group is constituted by sugars and water with an amount of 80% and 17% respectively [11]. The later group encompass the minor components of honey which are the phenolic compounds, minerals, enzymes, proteins, free amino acids, organic acids and vitamins. Flavonoids, anthocyanins, essential oils, sterols, pigments and phospholipids are also present. In the following paragraphs a comprehensively summary of the most important constituents is presented concerning their chemical structure, properties and significance for honey.

1.4.1 Phenolic compounds

1.4.1.1 Structure

Honey is comprised of a great variety of substances which come from various sources. Although the abundance of phenolic compound is not high compared to other constituents like sugars, they will be firstly and thoroughly discussed as the aim of this thesis is phenolic compounds determination. Phenolic compounds or polyphenols are a group of compounds which include a great variety of molecules with at least 8000 different known structures [12]. These compounds are secondary metabolites of plants, biosynthesized mainly for protection against stress and oxidative damage and transferred via the nectar to the honey. The phenolic compounds of honey can be categorized into two main classes, phenolic acids and flavonoids. In both occasions, there can be further classified.

To begin with, phenolic acids can be separated into two groups, the hydroxybenzoic acids derivatives and the hydroxycinnamic acids derivatives (figure 1). Gallic and protocatechuic acid belong to the hydroxybenzoic acid class, a group of compounds that has not widely studied for its nutritional properties. On the other hand, the main representatives of hydroxycinnamic acids are ferulic, coumaric and caffeic acid. It has to be noticed that hydroxycinnamic acids are usually found as glycosylated derivatives or esters of shikimic, quinic or tartaric acid [13].

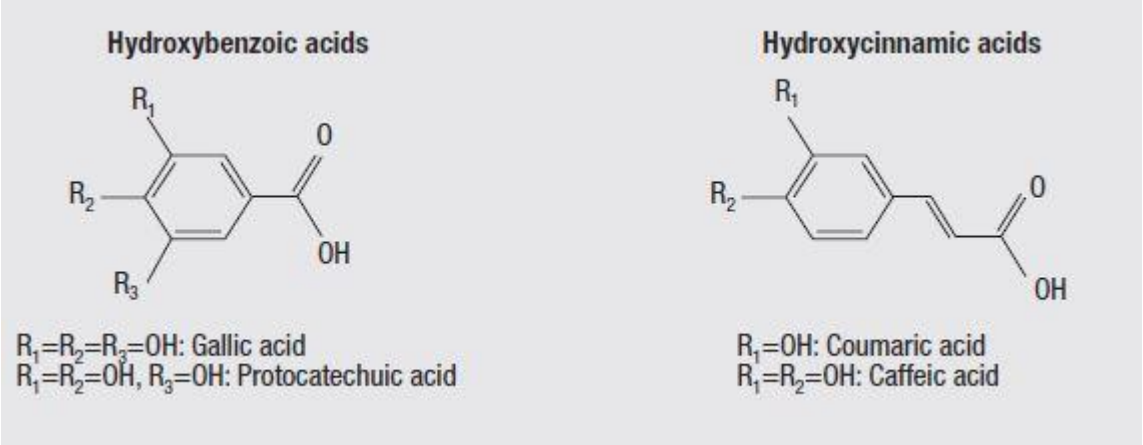


Figure 1. The structure of hyrdoxybenzoic and hydroxycinnamic acids, source: [13]

Flavonoids share a common carbon skeleton of diphenyl propanes, two benzene rings (ring A and B) joined by a linear three-carbon chain. The central three-carbon chain may form a closed pyran ring (ring C) with one of the benzene rings (figure 2).

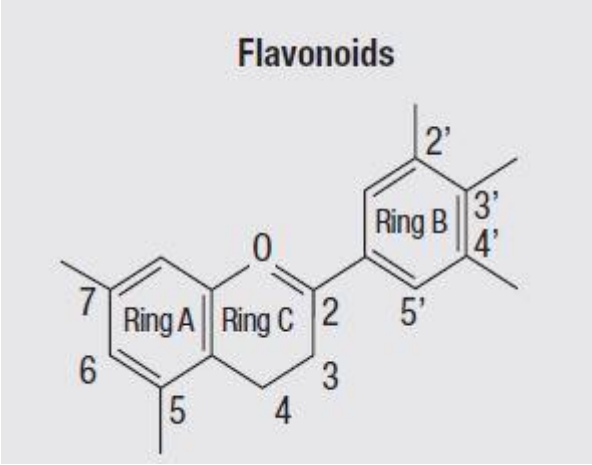


Figure 2. The structure of flavonoids, source: [13]

Flavonoids can be devided into flavonols, flavones, flavanones, isoflavones, anthocyanidins and flavanols (figure 3). More than 4000 flavonoids structures have been cited in literature and this number is constantly growing due to the occurrence of various substitution patterns in which primary substituents such as hydroxyl group can be substituted, like in the case of glycosylation, forming complicated structures with high molecular weight [14].

Flavonols have a double bond between C2 and C3, with a hydroxyl group in the C3-position. The most common flavonol in foodstuffs is quercetin. It has to be mentioned that the presence of flavonols is stimulated by light. Flavones have a double bond between C2 and C3, and are the less common flavonoids. Flavanones are characterized by the presence of a saturated three-carbon chain and an oxygen atom in the C4. They are generally glycosylated by a disaccharide in C7. Naringenin, hesperetin and eriodictyol are among the common observed flavanones. Isoflavones have hydroxyl groups in the C7 and C4 positions and they are also called phytoestrogens due to their structural similarity to estrogens. These compounds are thermosensitive and may be degraded to glycosides under thermal handling. Anthocyanidins are found mainly as glycosides of their respective aglycones form, with the sugar moiety mainly attached at the 3-position on the C-ring or at the 5, 7-position on the A-ring. Flavanols contain a saturated three-carbon chain with a hydroxyl group in the C3. Unlike other classes of flavonoids, flavanols are not glycosylated in foods. The main representative flavanols in fruit are catechin and epicatechin.

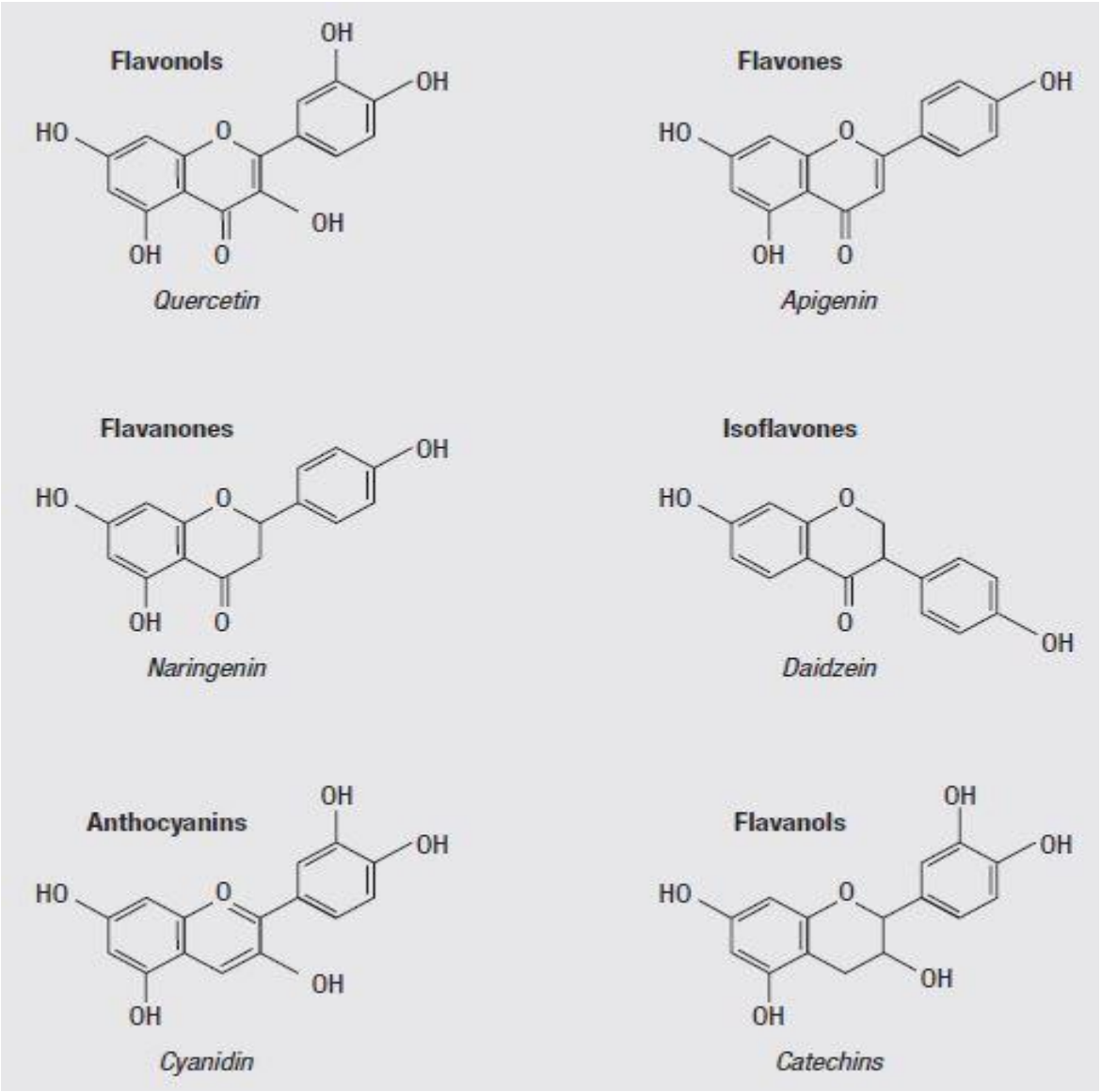


Figure 3. Structure of various flavonoids, source:[13]

1.4.1.2 Extraction

Honey is a complicated matrix which contains as main constituent sugars. This fact makes the determination of phenolic compounds a challenging task as the complexity of the matrix interferes with analytical procedure. Many efforts have been made for a high efficiency recovery rate during extraction of polyphenols in honey. Due to the wide range of polarities among the phenolic compounds, a suitable extraction procedure should be used in order to efficiently extract the compounds from the matrix.. So, three different approaches have been utilized for the extraction of phenolic compounds since now.

Firstly, Amberlite XAD-2 resin has been one of the most popular adsorbent media for the extraction of phenolic compounds from honey. The extraction procedure is well described in [15]. This extraction procedure shows high sensitivity for the phenolic compounds with recovery rates over 95% [12]. Nevertheless, there are several drawbacks that have to be highlighted. A high amount of honey sample, 50g or even 100 g, needs to be dissolved in water at a solvent to sample ratio of 5:1. Thus, a large quantity of honey is needed for this extraction protocol and as a result only samples with adequate amount can be analyzed. Proportionally, high volumes of solvents are also needed. One way or another, the current analytical trend is to minimize the volume of the sample in order to be both easily handled and because of the scarcity of some samples. The extraction with Amberlite XAD-2 comprises many different steps and can be considered as time consuming pretreatment. Moreover, this resin retains selectively the phenolic compounds. So, this pretreatment is not suitable for metabolomics studies in which a comprehensive fingerprint of the sample need to be obtained and the extraction procedure should be generic.

The second approach is quite similar to the previous one but diminish the previously mentioned drawbacks. Various SPE methods have been proposed in order to overcome the problems of the high volumes (solvents and sample) and time consuming procedure. In this case about 5 g of honey sample is required for the analysis. The phenolic compounds interacts with the adsorbent and retained by means of hydrophobic interactions. Therefore, a great variety of sorbents have been used such as Bond Elut octadecyl C18, Oasis HLB and Strata-X [16]. The cartridges are usually conditioned with methanol and acidified water. The polar substances are washed with water, while the polyphenols are commonly eluted with methanol. SPE combines the small amount of sample and reagents with high selectivity for phenolic compounds. Furthermore, the huge variety of sorbents give more choices for extraction. However,as the previous extraction protocol, you cannot obtain a comprehensive sample fingerprint due to the sorbent selectivity and thus this extraction method is best fitted for methods with a target approach.

Liquid-liquid extraction (LLE) is a classic approach in order to extract compounds, which exhibit affinity to the solvent used for the extraction, from a

matrix. Ethyl acetate (EtAc) is used as extractant in the most of the cases [17, 18]. It has to be noticed that repeated extractions are performed in order to achieve better extraction efficiency. Furthermore, LLE is also used as a preliminary clean up step during SPE procedure indicating that LLE is a more generic extraction which permits metabolomics studies [12]. Hence, the diluted-and-shoot approach has been recently proposed in order to obtain a fully representative polyphenols fingerprint [19]. In this case, the honey sample was analysed just after its solubilisation in water or in LC mobile phase. To sum up, SPE cartridges and LLE provided a simpler, less expensive and faster technique compared with that of Amberlite XAD-2 resin, which are also extensively used for the extraction of phenolic compounds in honey.

An innovative effort which utilizes the evolution of nanotechnology is the multiwalled carbon nanotubes (MWCNTs) sorbents for phenolic compounds [24]. MWCNTs are added to an acidified solution of honey, then the mixture is magnetically stirred in order to promote the retention of polyphenoles onto the nanotubes. The main advantages of this approach lie in the possibility to simultaneously extract a really wide number of phenolic compounds with high recoveries and reproducibility.

1.4.1.3 Analysis

The predominant technique for the separation of phenolic compounds is high performance liquid chromatography in reversed phase mode (RP-HPLC). Recent cases of developed HPLC methods for the quantitative and qualitative determination of phenolic compounds in honey are presented in table 1. All of the cited separations in table 1 are performed in C18 (ODS) columns highlighting the importance of this stationary phase for the separation of polyphenols. Moreover, the referred in table 1 methods were developed in the last 10 years in order to depict the analytical trend. Another advance in this field is the use of ultra-high performance liquid chromatography (UPLC) systems. Indeed, better separation is achieved because of the smaller size of the stationary phase particles (usually smaller than 2 μm) which permit more effective interaction with the analytes. According to Van Deemter equation the height equivalent theoretical plate (H) is inversely related to the particle size of

the column packing. Thus, reducing the stationary phase particle diameter an increase in number of theoretical plates are ensued resulting in better separation. These systems deliver the mobile phase at very high pressures resulting to better chromatographic and analytical features like resolution, sensitivity and accuracy. As regards the mobile phase used in RP chromatography, a mix of an aqueous solution acidified with formic or acetic acid and an organic solvent like methanol or acetonitrile is utilized. Another important issue for the determination of the phenolic compounds is the detection. Two different cases can be discriminated. The first is the measurement of the ultra violet (UV) absorption, sometimes performed using diode-array devices (DAD), while the other case is the use of mass spectrometry (MS). With respect to UV detectors, the selection of the appropriate wavelength in which most of the phenolic compounds absorb is an essential task. DAD detectors also provide the whole ultra violet – visible (UV-Vis) spectrum which add another indication for the identification of peaks. However, the major drawback of spectrophotometric detectors is lack of information about the structure of the molecules. The solution for this problem is the use of MS and tandem mass spectrometry or the combination of spectrophotometric detectors and MS. The fragmentation patterns obtained using MS or even better high resolution mass spectrometry (HR-MS) have changed the capabilities of identification of phenolic compounds. HR-MS instrumentation like orbitrap or quadrapole time of flight (QToF MS) is suitable for accurate determination not only of phenolic compounds but also the whole metabolomic fingerprint of the matrix. This is very important for authenticity studies where the discrimination of the botanical or geographical origin is the aim [20]. Moreover, electron spray ionization (ESI) in a negative mode is usually selected for the ionization considering the chemical structure of the polyphenols. In conclusion, M. Ciulu et al [21] have currently described in great depth the state of the art in the field and the reader can seek more information in this work.

Table 1. Recent chromatographic techniques for the analysis of phenolic compounds in honey

technique	stationary phase	mobile phase	floral/geographical origin	year	ref
UPLC-PDA-MS/MS	Nucleodur C18 Pyramid column (100 × 2.1 mm, 1.8 µm)	0.1% formic acid and 0.05% formic acid in methanol	Manuka honey from New Zeland	2012	[22]
LC-DAD-ESI/MS	Luna C18 (2) column (150 x 2.0 mm I.D x 3 µm particle size)	1 % formic acid and acetonitrile	7 botanical types of honey from Slovenia	2011	[23]
UHPLC–MS/MS Orbitrap	Syncronis C18 (100 x 2.1 mm x 1.7 µm)	0.1% formic acid and 0.1% formic acid in acetonitrile	sage honey from Croatia	2015	[24]
HPLC–DAD–MS	Eclipse XDB-C18 (250 x 3.0 mm ID x 5 µm)	1 % acetic acid and acetonitrile	buckwheat honey from Italy	2013	[25]
UPLC-Q/TOF-MS	Acquity UPLCTM BEH C18 column (2.1 × 100 mm x 1.7 µm)	2% acetic acid and methanol	5 botanical types of honey	2009	[17]
HPLC-UV	Varian Spherisorb ODS column (250 x 4.6 mm x 5 µm)	methanol and 20 mM potassium dihydrogen phosphate buffer	8 botanical types of honey from 8 countries	2007	[26]
HPLC-DAD	Nova-Pack C18 (150 × 3.9 mm x 4 µm)	5 % formic acid and methanol	unifloral and multifloral honey from Czech Republic	2010	[27]

1.4.1.4 Benefits using HR-MS techniques for the determination of phenolic compounds

As already mentioned above, LC coupled to MS is the most appropriate and promising analytical technique in phenolic compounds analysis. The development of high- resolution mass spectrometric systems (HR-MS) which includes mass analyzers such as time-of-flight (TOF), Fourier transform-ion cyclotron resonance (FT-ICR), Orbitrap or new hybrid approaches (e.g. Q-ToF), become the driving force in development of polyphenols identification methodologies because of their sensitivity and high mass accuracy. Three different analytical approaches for this procedure were presented by Krauss et al. [28]:

- Target analysis, a quantitative analytical approach using reference standards
- Suspect screening, a qualitative approach for candidate phenolic compounds without using reference standards
- Non-target screening, a qualitative approach for unknown polyphenols.

These workflows are depicted step by step in figure 4 and presented in the following pages. It has to be noticed that these approaches are firstly introduced in environmental analytical chemistry for the determination of the emerging pollutants [28, 29]. Afterwards, food analysis has also adopted these methodologies in order to elucidate the structure of important nutritive food constituents like polyphenols or harmful residues like pesticides. HR-MS is also a decisive factor for food authentication studies as a characteristic pattern of the selected analytes is obtained [30].

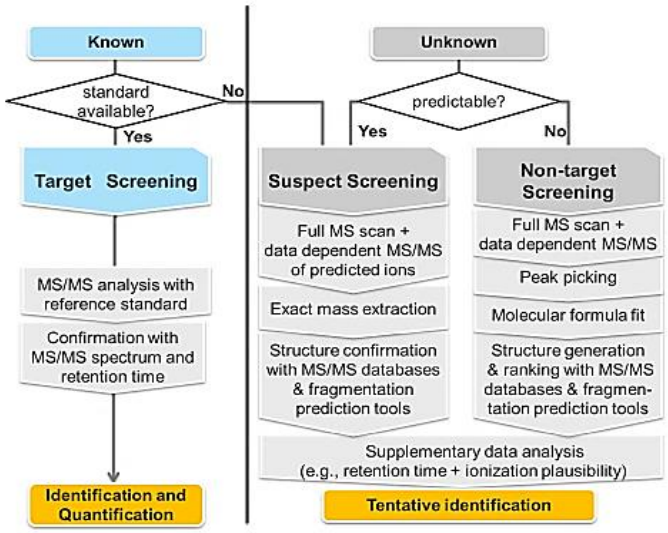


Figure 4. Flow chart of screening procedures. “Known” compounds have been confirmed or confidently identified before, other compounds are considered ‘”Unknown’”, source: [29].

1.4.1.5 Target analysis

Target screening approach, as shown in figure 4, referred to the detection and quantification of known compounds, which their reference standards are available and as a result their retention time and MS spectrum have already acquired. As a result, they can be included within a defined MS method and be monitored in routine analysis. LC combined with triple-quadrupole mass spectrometric detection (LC-QqQ MS/MS) is the prevailing technique for target analysis. The triple quadrupole (QqQ) analyzer permits application of MS/MS modes [e.g., production scan, precursor-ion scan, neutral-loss scan and selected reaction monitoring (SRM), which is the predominant]. The SRM mode provides several advantages and interesting characteristics for target analysis, such as increased selectivity, reduced interferences and high sensitivity, which allows robust quantification [19]. However, due to the fact that a predefined list of transitions have to be loaded in the MS method, only compounds from this list can be detected

The SRM limitations can be compensated by HR-MS target analysis. Practically, all compounds present in a sample that can be ionized in a specific ion source can

be detected simultaneously with HR-MS instrument operating in full scan mode, making it unnecessary to select particular compounds and their transitions. Target compounds included in an accurate-mass database are screened in the sample based on retention time (t_R), theoretical mass, isotopic pattern and MS/MS fragments [31]. Additionally, hybrid instruments have the option of data-dependent MS/MS acquisition, where MS/MS analysis is triggered if a compound from a target-ion list is detected in the full scan. Due to their high mass resolving power, these instruments improve the identification of isobaric compounds and thus permit a more reliable identification process for target analytes [32].

1.4.1.6 Suspect screening

Contrary to target analysis, suspect screening approach does not depend on reference standards for confirmation. Despite the fact that a large number of polyphenols do not have reference standards available, compound-specific information for suspected molecules, such as molecular formula and structure can be used for the identification and confirmation process. The molecular formula allows the calculation of the exact m/z of the expected ion which is in turn extracted from the high resolution full-scan chromatogram. In case of positive findings, several confirmatory steps must be followed in order to reach structure-derived information [33]. The exact mass for each of the predicted analytes is extracted from the chromatogram and checked by comparing it with control samples. An intensity-threshold value is applied to cut off unclear spectra. The plausibility of the chromatographic tR, isotopic pattern, and ionization efficiency are used as further filters to narrow down the number of candidate peaks. Furthermore, using MS/MS or MSn, structures of suspected phenolic compounds are suggested based on the observed fragmentation pattern and diagnostic fragment ions. Depending on the above criteria, there are different confidence levels of identification in HR-MS analysis.

1.4.1.7 Non-target screening

Non-target screening is the analytical approach for investigation of analytes which can be detected in the samples but no previous information is available. It is usually

performed after target analysis and suspect screening. Full identification of the non-target extracted m/z is a difficult task and for this reason HR-MS instrumentation is necessary in order to obtain high resolution data from full scan and MS/MS mode and elucidate reliably the detected m/z [34].

The assessment of massive quantities of data which offer HR instruments and finally the export of results require post-acquisition data-processing programs which offer rapid, accurate and efficient data mining. Thus a lot of open-source and commercial software exist, some of which are indicatively presented below:

- MZmine
- XCMS
- enviMass
- Bruker Metabolite Tools and Profile Analysis
- Waters MassLynx and MetaboLynx
- Thermo Scientific MetWorks.

The first and most critical step in non-target screening is peak peaking. This step gives the opportunity to exclude irrelevant peaks by the comparison of the sample with control or blank samples. Afterwards, the removal of noise peaks, mass recalibration, componentization of isotopes and adducts follow.

Exploration of online databases such as ChemSpider and PubChem or structure generation may lead to possible structures of the phenolic compounds. Also, information like molecular formula and substructures of the parent compound could be helpful for the purpose of the search restriction.

Even after filtering, strict criteria and thresholds, the number of peaks which correspond to non-target compounds is enormous and their interpretation would demand a great amount of effort and time. Therefore, the most intense peaks are chosen in order to be interpreted [35].

1.4.1.8 Structure elucidation and identification confidence levels in HR-MS

High mass accuracy coupled with high isotopic abundance accuracy is fundamental to elicit a reliable molecular formula generated by the software incorporated in the HR-MS instruments. The acceptable deviation of the experimental m/z from its corresponding theoretical of parent ions is usually defined at 5 ppm. This limit guarantees the correct prediction of their molecular formula. Higher errors, generally below 10 ppm, are acceptable in the workflow regarding their characteristic fragment ions. In spite of the fact that the accurate extrapolation of the elemental composition of a compound is essential, it is not sufficient to lead in a correct structure proposal.

A process which is very helpful in structure investigation is the observation of the presence or absence of similar characteristic ions in the fragmentation pattern comparing the data obtained and online spectral libraries. In addition, information from experimental MS/MS spectra can be compared with in silico mass spectral fragmentation tools (e.g. MetFrag, MetFusion, Mass Frontier, MOLGEN-MS and ACD/MS Fragmenter) or with mass spectra in libraries (e.g. MassBank and MetLin). Nevertheless, the use of mass spectral libraries is restricted for LC/MS-MS data because they do not have a great amount of available data and mass spectra of different instruments are not so comparable [29].

Consequently, the HR-MS based identifications of the analytes differ among studies and compounds because it is not always possible to synthesize each compound and confirm it. In order to make easier the communication of identification confidence , Schymanski et al. [36] proposed a level system which is described in figure 5.

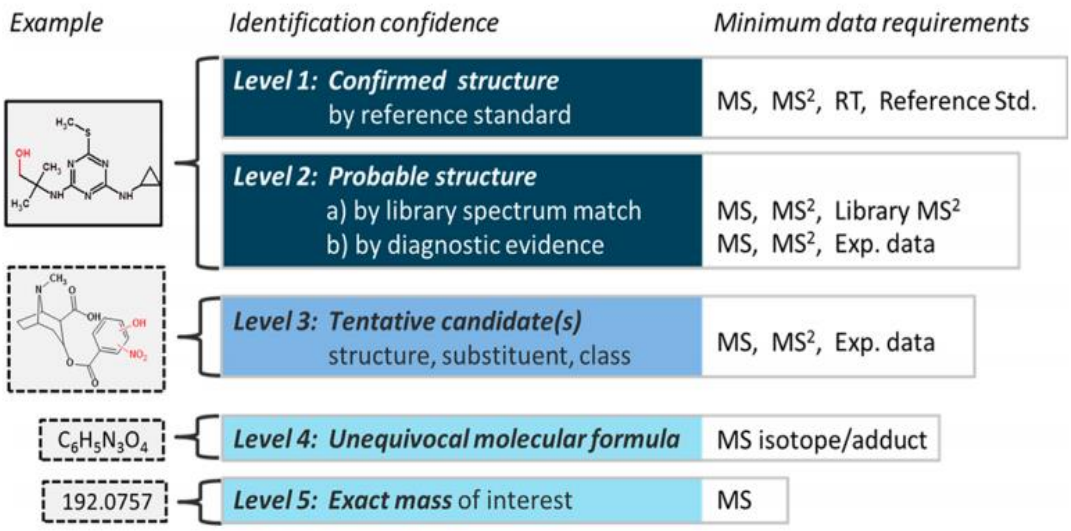


Figure 5. Proposed identification confidence levels in HR-MS analysis, source: [36]

- Level 1: Confirmed structure is the perfect situation where the candidate structure is confirmed by the measurement of a reference standard with MS, MS/MS and retention time matching.
- Level 2: Probable structure refers to a proposal for an exact structure based on different evidence.
    - Level 2a: Library which includes indisputable matching between literature or library spectrum data and experimental.
    - Level 2b: Diagnostic which refers in the case of no other structure fits in experimental data, but no standard or literature information is available.
- Level 3: Tentative candidate(s) is the situation where there is evidence for possible structure(s) but the experimental information is insufficient to the exact proposal.
- Level 4: Unequivocal molecular formula describes the case of an unambiguous formula which is assigned by the spectral information but there is no sufficient evidence to propose possible structures.

- Level 5: Exact mass (m/z) is detected in the sample but no experimental information exists in order to propose even a formula.

1.4.2 Sugars

Honey, as mentioned in section 1.1 , is a supersaturated sugar solution in which all kind of sugars may be found. The profile of sugars in honey has been studied globally. Generally, fructose, glucose, sucrose, rhamnose, trehalose, nigerobiose, maltose, isomaltose, maltotriose, maltotetraose, maltulose, melezitose, nigerose, melibiose, raffinose, palatinose, erlose and other carbohydrates have been found in several studies [37-39]. Concerning monosaccharides, fructose and glucose represent about 75% of the sugars found in honey, while the disaccharides sucrose, maltose, turanose, isomaltose, maltulose, trehalose are responsible for approximately 10-15%. Commonly, a rapid crystallization is observed in some honey types like rape (*Brassica napus*) and dandelion (*Taraxacum officinale*) in which the main sugar is glucose. In the great majority of cases the main sugar fraction in honey is fructose [40]. The concentration levels of fructose and glucose along with their ratio may be used as markers for the classification of monofloral honeys [10]. Additionally, trisaccharides such as maltotriose and melezitose have been measured in traces comparing to the other sugars. Moreover, disaccharides and trisaccharides may be hydrolyzed to monosaccharides. The hydrolysis is happened due to enzymatic activity. The most common example is the hydrolysis of sucrose to fructose and glucose by the enzyme invertase [41]. Some very important attributes like viscosity, hygroscopicity and granulation are directly connected to the presence of sugars. Moreover, nearly all of the energy value of honey are attributed to these compounds. Several factors affect sugar composition like geographical origin [42], botanical origin [43], processing [44] and storage [45].

1.4.3 Organic acids

Little attention has been paid to the organic acids (OAs) of honey until now due to the fact that they were found in honey in very small quantities, about 0.5% of its fresh weight. OAs presence is due to the derivatization of sugars by enzymes secreted by honeybees when transforming the nectar into honey. It has also

revealed an increase in acidity over time because of the transformation of sugars and alcohols into OAs by the action of honey yeasts [10]. Furthermore, OAs affect important properties of honey like color, flavor, electrical conductivity, potential of hydrogen (pH) and acidity. Free acid content is usually used for the determination of honey spoilage. However, this parameter can be measured in different ways resulting in significantly different results. Thus, OAs profile may feel the gap of a widely accepted method for a more appropriate determination of honey spoilage. Moreover, OAs provide antibacterial activity to honey, while they may be useful as fermentation indicators indicating a microbial spoilage. It is also important to notice that they have been used as alternative authenticity markers for the verification of both the geographical and botanical origin [46]. The most abundant OA in honey is by far the gluconic acid. Its occurrence originates from the activity of the enzyme glucose-oxidase on nectar glucose. High levels of gluconic acid are correlated with the duration needed for the total transformation of nectar into honey. The longer the duration, the higher the concentration of glucose-oxidase added by the bees, and hence the larger the amount of gluconic acid synthesized [47]. Furthermore, gluconic acid is produced by the metabolic activity of the bacteria belonging to the genus Gluconobacter spp. In fact, large quantities of gluconic acid are produced under aerobic conditions in the presence of high glucose concentrations. Other OAs found in honey are citric, levulinic and formic acids. These compounds may be originated as intermediates of the Krebs cycle or other enzymatic pathways. It has to be noticed that 5-hydroxymethylfurfural (5-HMF) can be transformed to one molecule of levulinic acid and one molecule of formic acid after a series of reactions increasing the concentration of free acidity in honey [48]. There are several techniques capable for the determination of OAs in honey.

The predominant technique is chromatography [49, 50]. Qualitative and quantitative measurements of OAs is regularly performed by HPLC and ion chromatography (IC) [51]. The analytical problem in this case is the matrix effect that may occur by the high concentration of sugar in honey matrices. Therefore, a solid-phase extraction may have to be performed as a cleaning step for the determination of OAs in honey. In the case of gas chromatography a derivatization

procedure is necessary as most of the OAs are non-volatile compounds. Chromatography is an efficient and accurate technique, however it is time consuming and non-economical. Hence, enzymatic methods solve this kind of problems as they feature simple instrumentation [52]. Moreover, they are specific and sensitive methods. Nevertheless, the main drawback of this technique is that it only measures one organic acid at the time.

1.4.4 Proteins, amino acids & enzymes

Proteins, amino acids and enzymes are not widely studied in honey. The reasons are both the small amount of proteins in honey, about 0.1 - 0.5% with molecular weights ranging from 22 to 75 kDa, and the difficulty to extract proteins from the sugar rich matrix. The presence of proteins in honey is mainly attributed to pollen. However, honeybees also add proteins through their pharynx and the nectar secretions.

A great variety of amino acids may be found in honey. In detail, more than 20 different amino acids, like proline, glutamic acid, phenylalanine, alanine, leucine and tyrosine, have been reported in honey [53]. In fact, proline is the most plentiful amino acid in honey and it is suggested as a criterion for the estimation of the maturation of honey. Moreover, adulteration of honey with sugar can be investigated checking the content of proline. An amount of at least 180 mg kg-1 of proline is accepted as the limit value for pure honey. Another important fact is the use of free amino acids as authenticity markers. Blossom and honeydew honey may be discriminated upon their free amino acids contents. Furthermore, specific amino acids have been cited as identical for specific botanical origin. Typical examples of that is the case of tryptophan for acacia honey and proline and phenylalanine for lavender honey [54].

Enzymes as a fraction of the proteins are also presented in honey. In detail, diastase, invertase (α-glucosidase), glucose-oxidase, catalase and acid phosphatase are the most prominent. The properties of these enzymes are already known [55]. Diastases consists of α- and β-amylase which are a group of starch

digesting enzymes. Particularly, α-amylase hydrolyses starch chains at random locations, producing a variety of dextrins, while β-amylase splits the reducing sugar maltose from the end of the starch chain. Regarding invertase, as it was mentioned before, it is responsible for the hydrolysis of sucrose to a molecule of fructose and a molecule of glucoce. As for glucose-oxidase, it has been found that it converts glucose into δ-gluconolactone, which is then hydrolyzed to gluconic acid, the most abundant organic acid of honey. Supplementary, glucose oxidase also produces hydrogen peroxide, which has proven to demonstrate anti-bacterial action.

Current methods for separation and quantification of proteins have comprehensively discussed in literature [56]. The common analytical choices, for the determination of the whole protein content, are Lowry and Bradford assays. However, these assays are time consuming and they are non-specific. Another widely used technique for peptide separation is gel electrophoresis like Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). A two dimensional gel electrophoresis is preferred in proteomic studies, where mass accuracy and resolution are indispensable. Moreover, the importance of protein separation and identification by LC-MS/MS has to be highlighted. The strengthening of analytical capabilities brought revolutionary progress in the field mainly in biological samples. This improvement is strictly connected to the development of sub-2-μm stationary phase for UPLC systems, the soft ionization techniques such as matrix assisted laser desorption ionization (MALDI) and ESI as well as the HRMS instruments like ToF. Finally, the investigation of honey proteome is a challenging field because of honey matrix, protein abundance, size and hydrophobicity and should be further studied.

1.4.5 Vitamins

Almost all of the vitamins in honey is water soluble, as expected, due to its aqueous nature containing a high quantity of sugars and an extremely low percentage of lipids. Especially, the B-complex vitamin such as thiamine (B1), riboflavin (B2), nicotinic acid (B3), pantothenic acid (B5), pyridoxine (B6), biotin (B8 or H) and folic acid (B9) are the most abundant. Regarding vitamin C, it is also exist in honey.

The vitamins are derived from the pollen grains and are preserved due to the low pH of honey [10]. The assessment of vitamins in foodstuffs is often a challenging issue due to their instability. In general, storage conditions, aging and processing are among the main reasons that make the determination of vitamins a difficult analytical process. Particularly in honey, the major obstacle is the commercial filtration procedure [57]. Honey is filtered in order to aesthetically please the consumer. Furthermore, it is ensured that the honey remains fluid for a longer period of time. Filtering removes bee parts, wax and solids, including the majority of pollen that can hasten crystallization. However, vitamins are also influenced and their content is dramatically reduced in the processed honey. Another important factor that complicates the measurement of vitamins is the enzyme glucose oxidase. This enzyme produces hydrogen peroxide which oxidize ascorbic acid causing vitamin loss.

Vitamins are essential compounds which enhance the nutritional value of honey. In fact, these nutrients do not penetrate into tissues and as a consequence there is no calorie income during their consumption. Thus, many foodstuffs are fortified with vitamins in order to be both nutritive and with low calories content. Unfortunately, analytical choices for the determination of vitamins in honey, unlikely to other organic compounds, are limited. Chromatography is the preferred technique in the most of the cases. Recently, an UPLC-MS/MS has developed for the determination of B-group vitamins [58]. Moreover, several RP-HPLC methods have been proposed using UV-Vis[57], FLD [59] and DAD[60] detector for the determination of group-B and vitamin C. Alternatively analytical options for vitamins measurement are near infrared spectroscopy (NIR) [61] and flow injection analysis (FIA) system with amperometric detector [62]. It has to be noticed that the developed analytical methods can only measure one or a few vitamins.

1.4.6 Minerals

Minerals are found in trace quantities in honey and their content ranges between 0.04% in light honeys to 0.2% in dark honeys. Macro- and trace elements such as potassium, magnesium, calcium, iron, phosphorus, sodium, manganese, iodine,

zinc, lithium, cobalt, nickel, cadmium, copper, barium, chromium, selenium, arsenic, and silver have been reported in various honeys [10]. These elements are important for nutritive (P, Mg, Ca) and health aspects (As, Cd, Cr). Although they are consider as a minor constituent their presence may indicate the botanical and geographical origin of honey [10]. To begin with, metal concentration is stimulated by the area that the beehive is located. Hence, every region has a different geochemical and geological profile. Consequently, the plants uptake different amount of metals based on both their metabolic needs and the soil individual characteristics. For instance, it has been revealed that apiaries located by the sea resulted to an increased value of K and Na in honey [63] . Additionally, botanical origin is a factor that influences the mineral content of honey. Acacia is a very resistant plant which can grow even under difficult climatic condition or drought. As a result the mineral uptake of this plant is very poor which is reflected to the produced honey. Taking into consideration the above mentioned facts, mineral content can be used in authenticity studies in order to discriminate the geographical and botanical origin[64].

Another issue that rules the amount of metals is the distance that bees can cover in order to forage. Bees can travel about 7 to 50 km^2 in order to find flowering plants. So even if the apiary is not located to a polluted area, bees may visit a polluted area resulting in increasing amount of heavy metals in honey. This is very important in the case of organically produced honey. The production of organic honey implies organic beekeeping which is defined in European regulation EEC No 2092/91, Annex I. The qualification of beekeeping products as being form organic origin is pertinent directly to both the characteristic of the hives treatments (e.g. application of veterinary drugs) and the quality of the environment. As all organic honey is produced in more or less polluted environment such contamination can never been eliminated. On the other hand, organic honey must not be contaminated by veterinary drugs introduced by bee keepers which are generally the most important contaminants [65].

Mineral content of honey can be measured with Atomic Absorption Spectroscopy (AAS), Inductively Coupled Plasma – Optical Emission Spectroscopy (ICP-OES) and Inductively Coupled Plasma – Mass Spectrometry (ICP-MS). Nevertheless, ICP-MS is the technique with the greatest potential. It features extremely low detection limits down to ppt level and multielement analysis including rare earth elements (REEs) determination. Recenlty, REEs have been successfully used as authenticity markers in various foodstuffs [66]. The main drawback of ICP-MS in comparison to AAS techniques is the cost for instrument purchase and the running cost in terms of consumables,i.e., argon and suprapur acids.

1.4.7 Volatile organic compounds

Volatile organic compounds (VOCs) comprise a group of compounds of high importance in honey. There is a great amount of identified VOCs in honey, more than 600 compound, which belong in many different chemical families [67]. In detail, hydrocarbons, aldehydes, ketones, alcohols, acids, furans and pyrans, benzenes and its derivatives esters, and terpenes. As it is widely known, VOCs are constituents which influence the aroma and the flavor of a foodstuff. It has been proved that every unifloral honey exhibit a distinctive aroma derived from the nectar and the plant and as a consequence from specific VOCs. The content of VOCs may also be influenced by processing conditions and storage as this type of molecules are thermosensitive. Additionally, even bees may produce or transform plant compounds resulting in compounds with volatile properties. It has to be highlighted that there are specific VOCs that are used as floral markers like hexanal and heptanal in lavender honey or 3,9-epoxy-1-þ-mentadieno, t-8-þmenthan- oxide-1,2-diol and cis-rose in lemon honey [10]. Except the floral markers, VOCs may also be used as indicator of honey freshness. A typical example of that are alcohols with methyl groups like 2-methyl-2-buten-1-ol and 3-methyl-3-butene-1-ol.

Regarding the analysis of VOCs in honey, GC-MS or GC-MS/MS is the ideal choice in order to quantitatively and qualitatively measure volatile molecules. However, there are several choices for the extraction of VOCs from the complex

honey matrix. Firstly, there are distillation methods like hydrodistillation which efficiently extract the VOCs. Nevertheless, these methods may lead to the formation of furan and pyran derivatives due to the effect of heat on sugar or amino acids [68]. LLE or ultra-sound extraction (USE) using non-polar extractants, like n-hexane, dichloromethane, diethyl ether, are also capable for selective extraction as they do not mixed with the polar substances of honey like sugars. The last analytical tries in order to reduce the use of solvents and to reduce the loss of VOCs due to heating are solid phase micro-extraction (SPME) and headspace (HS) methods. SPME has the advantage of being a flexible, simple and a relatively economic extraction technique. On the other hand, HS is performed directly to the sample. In this way, valuable time is saved and the VOCs profile is obtained as it occurs in the matrix. The use of HS SPME is also mentioned in order to achieve a better extraction efficiency [69].

1.5 Physical characteristics

Honey is a supersaturated aqueous solution which is mainly consists of simple sugars or polysaccharides and solids like pollen. The physical properties of honey influence decisively its quality. These are density, viscosity, hydroscopicity and granulation. The density is related to the water content of honey. In other words, a low amount of moisture results to a more viscous state. Viscosity of honey influences beekeepers during the production of the product. As the viscosity of honey increases, both the extraction procedure from the comb and the filtration become harder. However, the heating of the honeys is the solution for these kind of problems. Special attention should be paid on the heating temperature as honey includes various sensitive compounds like flavonoids and vitamins which strongly affect the nutritional value. On the other hand, a high moisture level may cause the fermentation of the honey. In detail, hydroscopicity is the capacity of honey to increase its moisture content when the humidity of the environment is higher than the products'. However, this is a reversible property as honey moisture may decrease if the environment is dry enough.

Granulation or crystallization of honey is of great importance. There is a misconception by the consumers that granulation of honey is linked to inferior quality. Absolutely different from what it is believed, honey crystallization is a natural process and happen due to the fact that it is a supersaturated solution. Thus, glucose tends to precipitate out of solution and the solution changes to the more stable saturated state. In fact, monohydrate glucose molecules are the initial points for the formation of crystals. There are several factors that pose impact on crystallization. Some batches of honey never crystallize, while others do so within a few days of extraction. It is generally accepted that honey removed with extractors and pumps is likely to crystallize faster than if it was left in the comb. Most liquid honey crystallizes within a few weeks of extraction. The tendency of honey to crystallize depends primarily on its glucose content and moisture level [70]. Additionally, the stimulation of granulation is influenced by any small particles--dust, pollen, bits of wax or propolis, air bubbles--that are present in the honey. Moreover, storage conditions such as temperature and moisture may also influence the tendency of granulation. A noticeable fact is that honeys with less than 30% glucose resist to crystallization.

1.6 Organoleptic characteristics

1.6.1 Color

Color is the first attractive characteristic of honey, and as such is very important for the product profitability. Mass-produced honey is, usually, a blend made from many honey sources, and so may be uniform in color. But individually harvested honey can have a range of colors depending on the season, the nectar source, the time between nectar collection and honey harvest, and production details. It is an important parameter in the quality, acceptance and preference of consumers [10]. Honey's color is a controversial issue and there are many different opinions. To begin with, it is suggested that the color is due to the presence or absence of carotenoids, while others claim that polyphenols are responsible. Another possibility is a chemical caramelization of the saccharides in honey, catalyzed by the acids present in honey. Additionally, a Malliard reaction, happening between

the sugars and the amino acids of honey, may play role in the hue of the unifloral honey [71]. Whatever the source of the color, it is generally true that the darker the honey, the more intense the flavor. Honey experts know that its color can vary from light tones to almost black amber tones, with the most common being bright yellow, reddish or greenish. In many countries, the price of honey is related to its color. For this reason, the determination of color is a useful classification criterion for unifloral honeys.

Color classification of honey is globally made using the Pfund color scale. This method is proposed by the United States Department of Agriculture (USDA) and it is based on the comparison of honey color to a standard colored glass [72]. The color is measured using the Pfund colorimeter which has a simple instrumentation. The reference unit is the Pfund scale ranging from 0 to 140 mm. Initially the scale corresponds to very light-colored honey and increases up to the darkest honey (Image 1). Nevertheless, the method cannot distinguish small variations of color while the determination is time consuming, needs a great amount of sample and it is significantly influenced by the operator as each individual observe in a different manner the hue of the color. In this way, different approaches have been proposed in order to accurately assess honey color. Tristimulus method can be considered as a more objective one [73]. However, this method has not been adapted in routine quality control. An innovative approach has recently proposed by Marina A. Dominguez and María E. Centurión [74] who apply digital image analysis combined with chemometric tools for determining honey color. This effort provides more reliable and faster determination compared to the reference method, while less sample is required.

Figure 6. The Pfund color scale, source: https://britishhoney.com/pfund-colour-scale/, last accessed 4/1/2017.

1.6.2 Flavor and Aroma

Consumer preferences for a specific product can be directly linked to its flavor and aroma. These factors show a great variation which is directly connected to the botanical origin of the product. Additionally, they can also be affected by processing conditions and storage time [10]. In the case of unifloral honeys, flavor and aroma can be considered as a unique characteristic because of their certain composition [75]. Consequently, the presence of specific compounds provide a different organoleptic result. To begin with, sugars, acids, and other volatile components like C1–C5 aldehydes and alcohols have a major impact on flavor and aroma. In fact, the sugars are the main compounds affecting taste. It is widely believed that honey with a high fructose content, like acacia unifloral honey, are sweeter compared to those with high glucose concentration such as rape unifloral honey [3]. On the other hand, aroma is mainly attributed to acids. Carboxylic acids are chemical compounds that have different aroma, ranging from spicy to rancid

depending on the length of carbon chain. Short chain acids such as acetic acid, have a spicy aroma, while butanoic acid and hexanoic acid, which are usually found in butter, are linked to a rancid aroma. Moreover, many phenylacetic esters have a honey-like taste and aroma while methyl and ethyl formate have been identified in honey [67]. Another factor that surprisingly affects organoleptic characteristics is the mineral content of the honey. It has proved that high mineral content is proportional to a darker color and a stronger flavor [76].

1.7 Quality criteria

Nowadays, food safety is an unquestionable issue, especially in the western world. Attention should be paid on quality because consumers' standards are high. In fact, they are willing to spend a lot of money for qualitative foodstuffs. In EU, where quality schemes are defined (PDO,PGI) certified products are much more preferred than common even though their price are higher [77]. Quality includes all the features that confirm a product's value to the consumer. This comprises negative attributes such as spoilage, contamination with filth, discoloration, off-odors and positive attributes such as the origin, color, flavor, texture, processing method of the food [78]. In the case of honey, Council Directive, 110/2001/EC governs all quality criteria of honey, while authenticity issues are defined internationally by the Codex Alimentarius. According to the legislation, the parameters that should be tested in order to assure quality of honey are sugars content, moisture content, water insoluble content, electrical conductivity, free acidity, 5-HMF and diastase activity. The accepted values of each compound are well defined and if a product does not meet them, then it cannot be placed on the market. Among the above mentioned quality markers sugars content, moisture content, 5-HMF and diastase activity are essential for the quality control of honey. In this way, these four parameters are discussed while in table 2 all quality criteria are established alongside with acceptable values and analytical techniques used for their determination.

1.7.1 Sugars content

Thermal processing and storing of honey do effect the composition of sugars. In these cases, furans like furfural, which is derived from pentoses, and 5-HMF, derived from hexoses such as glucose and fructose are formed. Consequently, these compounds have strongly connected to an undesired product as there is a degradation in quality. Finally, storage results in a slight decrease , about 15%, in the quantity of fructose and glucose due to the acid catalyzed formation of maltose and other reducing disaccharides [71].

As already mentioned in the section 1.4.2, honey is mainly constituted of sugars. In this way, legislation stipulates that fructose and sucrose should be over 60 g 100 g^{-1} and 45 g 100 g^{-1} for floral and honeydew honey respectively (table 2). It has to be mentioned that sugars composition is affected by the botanical and geographical origin. Therefore, if a different amount of reducing sugars is detected then a fraudulent practice should be considered possible. There is a wide range of constituents that are used in honey fraud. To begin with, high fructose corn syrup (HFCS), sucrose syrups and glucose syrups are commonly detected as adulterants. These compounds come from beet, canes and invert-sugar syrups. In fact, the key for the detection of adulteration is the amount of fructose and glucose. However, the addition of the syrups does not mean a radical change in composition. A typical example of that is the adulteration with HFCS which is hardly revealed. Fraudsters are always trying to find new adulterants in order to increase their profit. In this way, rice syrup is recently used in this way. This kind of fraud detection is very challenging because rice and honey producing plants are C3 plants. In other words, these plants follow a similar Calvin cycle of photosynthesis resulting to a similar carbon isotopic fingerprint. On the other hand, the detection of cane sugar is a common practice because cane belongs to C4 group and have a different carbon isotopic fingerprint [79]. Another important issue is the indirect adulteration of honey. Honey is a natural product meaning that honeybees are exclusively fed by their own. However, beekeepers are usually feed their colonies in order to increase honey production. This fact has unknown impact on sugars composition.

Recently, L. Wu etc [80] have thoroughly discussed the available analytical methods in order to detect honey adulteration. Firstly, stable carbon isotopic ratio analysis (SCIRA) feature the predominant choice. This fact arises from the altered isotopic ratio ($^{13}C/^{12}C$) that is observed between plants with a different photosynthetic pathway. Chromatographic methods like GC and HPLC are also used with a variety of detectors such as MS or refractive detector index (RDI). The utilization of GC allows the detection of difructose anhydrides (DFAs) and HFCS due to the high sensitivity of the technique. The DFA content may be used as a marker in order to check food composition and processing. DFAs are formed during heating of sugars or intense thermal processing of foodstuffs with high amount of sugars. It has to be noticed that the main fraction of DFAs is not volatile. According HPLC methods, high performance anion exchange chromatography (HPAEC) is capable for the separation of every sugar fraction based on structural differences of mono-, oligo- and polysaccharides. Additionally, HPLC with a reflective index detector (RDI) is also a very selective, economic and precise choice for the determination of starch syrups. The use of UPLC-Q-ToF MS provide accurate determination of different sugar syrups like corn syrup and rice syrup. A huge amount of data are emerged through the abovementioned techniques. The treatment of this results with chemometric tools is of indispensable importance in order to accurately detect the fraudulent practices. Common chemometric tools used for this purpose are principal component analysis (PCA) or partial least squares (PLS) data compression methods transforming the data set into a reduced set of new variables.

1.7.2 5-HMF

5-HMF is widely recognized as an indicator of honey freshness. It is formed by the decomposition of monosaccharides or the Maillard reaction, when honey is heated or stored for a long time [81]. A proposed mechanism for the formation of 5-HMF in honey is illustrated in scheme 1. The increase of this compound is directly related to a high heat treatment temperature and a long storage time. Consequently, old honeys contain highest concentrations of HMF than fresh

honey. Additionally, several studies, which are comprehensively reviewed by C.P. Calvo and M. Vazquez [7], indicate that there is a greater concentration of 5-HMF in honeydew honey than in blossom honey. 5-HMF content is stipulated by the EU Directive 110/2001 in order to ensure the quality level of honey. In detail, the upper eligible concentration for 5-HMF in honey is about 40 mg/kg. However, there are two occasions with different limits. The first case is honey produced from countries or regions with tropical temperatures. The reason is that high temperature increases 5-HMF concentration and consequently the limit is doubled (80 mg/kg). Moreover, honey with a low enzymatic level has an eligible 5-HMF content that should not exceed 15 mg/kg. Indeed, a qualitative honey is expected to have a low HMF content. 5-HMF can only indicate overheating or inappropriate storage conditions as other factors like sugar profile, presence of organic acids, pH, moisture content, water activity and floral source may influence its content. It should be noted that there is a chance for 5-HMF formation in low temperatures in acidic conditions by dehydration of sugars [82]. Finally, analytical methods for the determination of 5-HMF were well established and described [10, 83]. Methods based on chromatographical principles are the more common analytical choice.

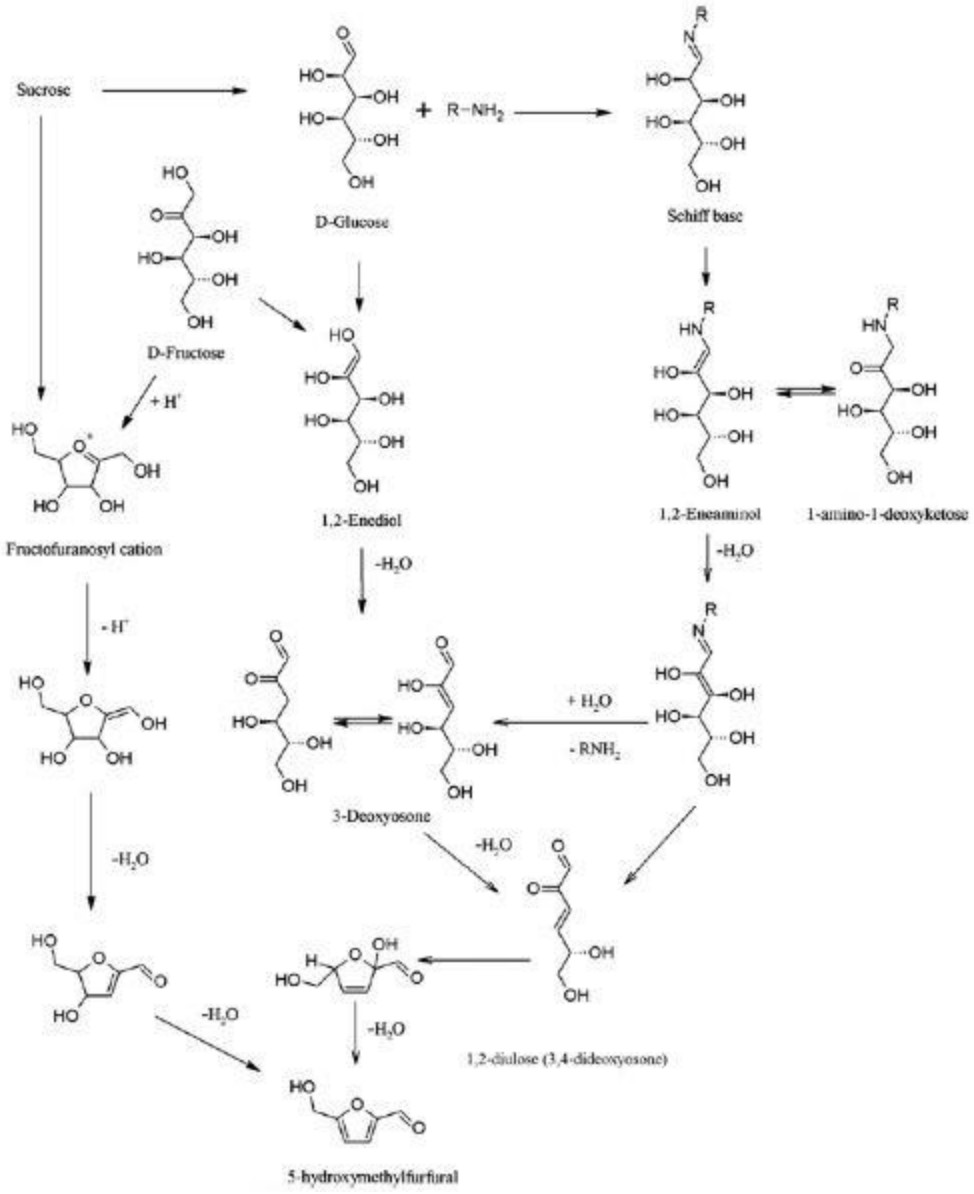


Figure 7. The formation of 5-HMF in honey, source: [9]

1.7.3 Moisture content and water activity

The importance of moisture content of honey should be underlined as it greatly affects the quality of the product. Water is among the main compounds of honey and it represents on average about the 17%. According to the Council Directive 2001/101/EC in general the moisture content of honey should not exceed the 20% of the total product. However, moisture content is not stable as honey shows a high hydroscopic property, in other words it can easily absorb water under certain conditions. Therefore, moisture content changes alongside the environment that surrounds honey. Under high relative humidity conditions, it is proved that there is an increase in moisture content [84]. Indeed, storage of honey should be taken place under a dry environment in order to assure that there is no any spoilage by an emerging fermentation. Nevertheless, it is not the moisture content which governs and controls the microbial growth but water activity. Water activity represents the water content which is “available” for the microbial growth. Water molecules are mainly binded by soluble solids. Consequently, microorganisms have not enough moisture in order to grow. Honey contains osmophilic and osmotolerant yeasts that can produce ethyl alcohol affecting the quality of the honey [85]. In the case of crystallization, the soluble solids are reduced and the water activity is increased so there is a greater chance for the degradation of quality. Honey usually has a water activity between 0.50 and 0.65. In Food Science a water activity values under 0.60 presents a stable environment where microorganism cannot survive. Moreover, moisture content is depended on the level of maturity achieved in the hive, the region, the climate and the production season while physicochemical properties like viscosity and crystallization are affected also by these factors [42, 73]. The determination of moisture, using a refractometer, is a routine procedure in honey analysis. It has to be noticed that this kind of measurement is not a precise one and in order to access accurately the moisture content a Karl-Fischer titration should be performed [86].

1.7.4 Diastase activity

As already mentioned, diastases include α- and β-amylase which are a group of starch-digesting enzymes. Alpha amylase degrades starch to a mixture of the disaccharide maltose, the trisaccharide maltotriose and oligosaccharides known as dextrins. Diastase activity comprises another option for the evaluation of honey quality. Anolgside with 5-HMF content, a decrease diastase activity can indicate excessive aging or thermal processing in a temperature over 60 ^{o}C. EU Directive 110/2001 poses as a minimum of diastase activity equal to 8 diastase number (DN) in Schade units for the majority of the honeys. In detail, diastase activity is expressed as the DN in Schade units. Particulary, one diastase unit is related to enzymatic activity which can hydrolyse 0.01 g of starch in 1 h at 40 ^{o}C according to 1 g of honey. However, there is an exception for honeys with low enzymatic activity and the permissible value of DN units is over 3. Honey produced from young nectars in early spring is the reason for a lower enzymatic activity. At this time of the year the sugar content is higher as the bees are not enough enzymatic active. Moreover, the age of the bees may be a decisive factor. Diastase activity can be determined using the classical Schade procedure or by the commercial Phadebas tablets [87]. The Schade procedure is a spectrophotometric method which uses a standard starch solution. The starch solution is treated with triiodide and produces a blue color. Then the starch solution is mixed with the honey, and the enzymatic activity of the sample reduces the intensity of the blue color. This reduction of the color is successively measured at 660 nm. However, Schade procedure is a quite slow determination while there are consecutive dilution which can increase the associated error of the method. For this reason, there are current efforts trying to improve the needed time for analysis and fully validate the results in order to obtain accurate results [88].

Table 2. Quality criteria of honey according the EU Directive 110/2001/EC

quality criterion	type of honey	acceptable value according to110/2001/EC
fructose and glucose content	blossom	> 60 g/100 g
	honeydew blends of honeydew honey with blossom honey	>45 g/100 g
sucrose content	in general	> than 5 g/100 g
	false acacia, alfalfa etc	> 10 g/100 g
	lavender, borage	> 15 g/100 g
moisture content	in general	< 20 %
water-insoluble content	in general	< 0,1 g/100 g
	pressed honey	< 0,5 g/100 g
Electrical conductivity	honey not listed below, and blends of these honeys	<0,8 mS/cm
	honeydew and chestnut honey and blends of these except with	>0,8 mS/cm
	those listed below not more than 0,8 mS/cm	
	exceptions: strawberry tree, bell heather , eucaluptus	
	lime (Tilia spp.), ling heather, manuka, tea tree	
Free acidity	in general	< 50
	baker's honey	< 80
Diastase activity	in general, except baker's honey	>8
	in general, except baker's honey	>3

HMF	in general, except baker's honey	< 40 mg/kg
	honeys of declared origin from regions with tropical climate and blends of these honeys	< 80 mg/kg

1.8 Health benefits of honey

Honey has not been used only as a foodstuff but also as a medicine since antiquity. Its beneficial properties are linked to burns, ulcers and wound healing[89]. However, scientific community has not researched the protective and therapeutic impact in depth until recently. This research provided that honey consumption protects from cardiovascular diseases, cancer and microbial infections [3]. The health benefits of honey emerge from the great variety of its compounds. In fact, honey features many antioxidant agents like phenolic acids and flavonoids which enhance the antioxidant, antiinflammatory, antimutagenic, antimicrobial activities both. In this subchapter, the antioxidant capacity of honey is critically discussed while the main health beneficial properties of honey are briefly summarized.

1.8.1 Antioxidant capacity

The term “oxidative stress” describes the lack of equilibrium between the production of free radicals and the antioxidant protective activity in a given organism. Antioxidant capacity (AOC) or antioxidant activity is the ability and potential of honey to reduce oxidative reactions within the food systems and human health. Particularly, these oxidative reactions can cause harmful reactions in food products such as lipid oxidation and adverse health effects like chronic diseases and cancers. Honey is a foodstuff with a great variety of antioxidants. In detail, flavonoids, phenolic acids, enzymes, ascorbic acid, organic acids, carotenoid-like substances, amino acids, Maillard reaction products, and proteins. According to [90], AOC varies greatly depending on the botanical origin of honey due to different content of plant secondary metabolites as polyphenolics. Moreover, AOC is also associated with honey color. The darker the color, the greater the AOC of honey. Radical scavenging capacity is another important antioxidant property of honey (figure 8). It has been proved that honey is capable of scavenging hydroxyl and superoxide radicals resulting to protection of lipid peroxidation.

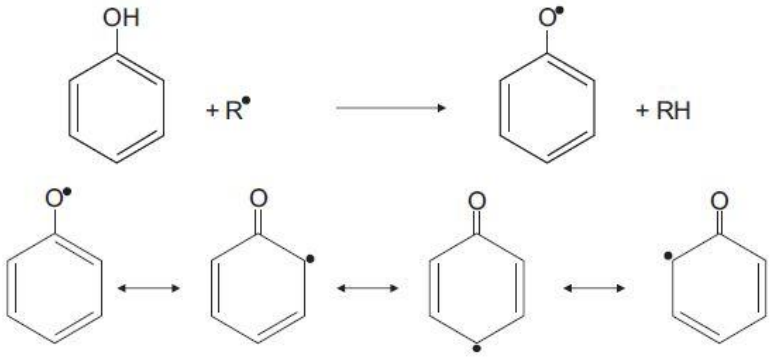


Figure 8. Radical scavenging mechanism of phenolic compounds, source: [89]

It is generally accepted that phenolic compounds greatly affect the AOC in honey. Thus, it is important to know how these molecules interact with radicals and which show the greatest antioxidative potential. To begin with, gallic acid has the better antioxidant properties among the hydroxybenzoic acids. AOC is improved with the increased number of OH groups in aromatic ring. Consequently, benzoic acid (1 OH group) has a decreased AOC compared to gallic acid (3 OH groups). On the other hand, hydroxycinnamic acids feature a stronger free radical scavenging ability. This characteristic appears to be linked to the inclusion of the unsaturated chain bonded to the carboxylgroup as a distinctive structure which provides stability by resonance to phenoxyl radical, even offering additional sites for the attack of free radicals [91]. Flavonoids have also impressive AOC. The presence of certain hydroxyl groups in the flavonoid rings increases antioxidant activity. Substitution patterns in the A ring and B ring, and the 2,3-double bond (unsaturated) and 4-oxo group in the C ring also affect the antioxidant activity of flavonoids. Nevertheless, the glycosylation of flavonoids decreases their antioxidant activity when compared to the corresponding aglycones [10].

There is a great variety of analytical methods for the determination of antioxidant activity. These methods are cited both in many review [92, 93] and original articles [94, 95]. For this reason there is no need for further discussion and the principles of these measurements are summarized in the following table 3, as is described by J. Alvarez-Suarez et al [96].

Table 3. Assays for the determination of AOC, source: [95]

Principle	Assay	Principle of Measurement	Quantification
Electron transfer	TEAC	ABTS•+ radical cation is reduced by antioxidants, causing absorbance decrease at 734nm	Trolox equivalents (mM TE/ 100g of honey)
	DPPH	DPPH• radical is reduced by antioxidants, causing absorbance decrease at 515nm	EC_{50}, RSE, Trolox equivalents (mM), ascorbic acid equivalents (mg/100 g of honey)
	FRAP	The ferric 2,4,6-tripyridyl-*s*-triazine complex is reduced by antioxidants, causing absorbance increase at 593nm	Ferrous ions equivalents, Trolox equivalents (mM TE/ 100g of honey)
Hydrogen atom transfer	ORAC	Free radicals are produced by AAPH and the fluorescent indicator protein *β*-PE is subsequently oxidized. Fluorescence is measured with emission and excitation wavelengths of 565 and 540 nm	Trolox equivalents (μmol TE)/gram of honey

1.8.2 Benefits from the consumption of honey

Honey is a foodstuff that can play a decisive role for a balanced nutrition. This nutritive character originates from the various compounds of honey that have been already discussed in this thesis. The health benefits from the consumption are numerous and well discussed in the literature [3, 90, 97]. The mechanisms and the reasons for each beneficial property is out of the scope of this thesis but in order to fully describe the state of the art the main health benefits from the consumption of honey are presented in the following bullets.

- Cardiovascular Diseases
    - Inhibition of inflammation
    - Improvement of endothelial function
    - Improvement of plasma lipid profile

    - Increase of low-density lipoprotein (LDL) resistance to oxidation
- Cancer
    - Antimutagenic capacity
    - Induction of apoptosis
    - Antiproliferative effect
    - Cytotoxic effect on several cancer cell lines
    - Antimetastatic effect
- Hypertension
    - Reduction of systolic blood pressure and malondialdehyde (MDA) levels
    - Ameliorament of susceptibility of kidneys to oxidative stress
- Diabetes
    - Reduction of glycaemia
    - Reduction of serum fructosamine
    - Reduction of glycosylated hemoglobin concentration
    - Attenuation of post-prandial glycemic response

CHAPTER 2
Honey authenticity

2.1 Introduction

Consumers always have a strong desire to know the origin and composition of the foodstuffs that they buy. Currently, especially in the western world, consumers want to know unequivocally the geographical origin and the content of a food. In fact, they prefer to spend more money in order to purchase a product of high quality. Labeling legislation dictate the necessary compounds and criteria that should be listed on the package. Thus, consumers are fully informed about the components and the additives of the foodstuffs. On the other hand, the regulatory bodies are responsible to develop analytical methodologies in order to assure that the label of a product comply with specifications.

Authenticity means the quality of being authentic or genuineness (www.dictionary.com, last accessed 03/2017). This word originates from the Greek word authentikós which means original, primary, at first hand and it is equivalent to authént (ēs) which means one who does things himself. Similar definitions which describe authenticity are genuineness and truthfulness of origins, attributions, commitments, sincerity, and intentions. Food authentication is the process that verifies that a food is in compliance with its label description [98]. This may include, among others, the origin (species, geographical or genetic), production method (conventional, organic, traditional procedures, free range), or processing technologies (irradiation, freezing, microwave heating). Individuals like importers, exporters, consumers and organizations including the scientific community, law enforcement authorities, and food producers are significantly interested in food authentication. The interest for food authenticity reached unprecedented levels and continue to grow, so this field attracts high-level attention from authorities and media around the world [20]. The assurance of existence of specific quality

attributes in high-value products like honey, is of particular interest since these products are susceptible to fraudulent techniques.

As regards honey, the annual world production is about 1.2 million tons. In the European Union, which is both a major honey importer and a producer, the annual consumption per capita varies from medium (0.3–0.4 kg) in Italy, France, Great Britain, Denmark and Portugal to high (1–1.8 kg) in Germany, Austria, Switzerland, Portugal, Hungary and Greece, while in countries such as the USA, Canada and Australia the average per capita consumption is 0.6–0.8 kg/year [97]. Taking into consideration the above mentioned factors, honey authenticity is an issue of great importance. Consequently, honey adulteration is motivated by the profit.

According to the Swiss Bee Research Centre [99], authenticity of honey can be divided into two separate aspects. The first one is referred to honey production type while the second one is referred to geographical and botanical origin of honey. The first case is linked to the processing of honey during the production procedure. This processing include centrifugation, filtering and pasteurization. Centrifugation is used in order to extract the honey from the combs, while filtering is performed for the removal of pollen, wax or other undesired compounds. Particularly, filters with a mesh size not smaller than 0.2 mm have to be utilized. Nevertheless, a common fraudulent practice is the use of smaller filters in order to filter out various undesirable contaminants without the notice “filtered” on the label of the final product. Pasteurization, under standardized circumstances (7.5 minutes at 63 ^{o}C or for 1 minute at 69 ^{o}C), is needed for the deactivation of osmotic yeast. However, an extended thermal handling will result to decrease in honey’s quality because of the loss of VOCs and nutritive compounds like polyphenols and vitamins. Consequently, all of these processing actions should be clearly mentioned on the package. Moreover, the addition of syrups or water are also deceitful actions focusing on the minimization of the cost production and increased profit [78].

The geographical and botanical origin that is mentioned on the label are two factors of great significance as the final price is decisively influenced by them. A typical example of this is that in many European countries the honeydew honeys like fir

honey are more expensive due to consumers' preferences[7]. Furthermore, geographical origin misleading description is also an important authenticity task. In this way, the imported honey from China or South America usually features a lower price. In these countries, especially in China, various honey fraud incidents have emerged resulting in a bad reputation [100]. Differences in price exist between countries in Europe and even between geographical regions inside a country. To this end, the thesis aims to report an up to date view on honey authenticity regarding the legislation of geographical indications (GIs), the techniques used for the determination of the origin and the potential authenticity and discrimination markers.

2.2 Scientometric evaluation of the field

Nowadays, determination of geographical and botanical origin, adulteration and mislabeling of honey are among the main aspects in food authenticity. In this way, a scientometric evaluation of the field has been demonstrated. Scopus database has been examined in order to reveal research trends. The following keyword was used "(authentication OR "geographical origin" OR authenticity OR "food authentication" OR "food geographical origin" OR "organic food") AND honey" resulting to 452 results. However, only about 200 publication were strictly connected to honey authenticity after a critical assessment of the result. Consequently, published research, as depicted in figure 9, are growing exponentially after 2008. This fact reflects that scientists have become more conscious about honey authenticity.

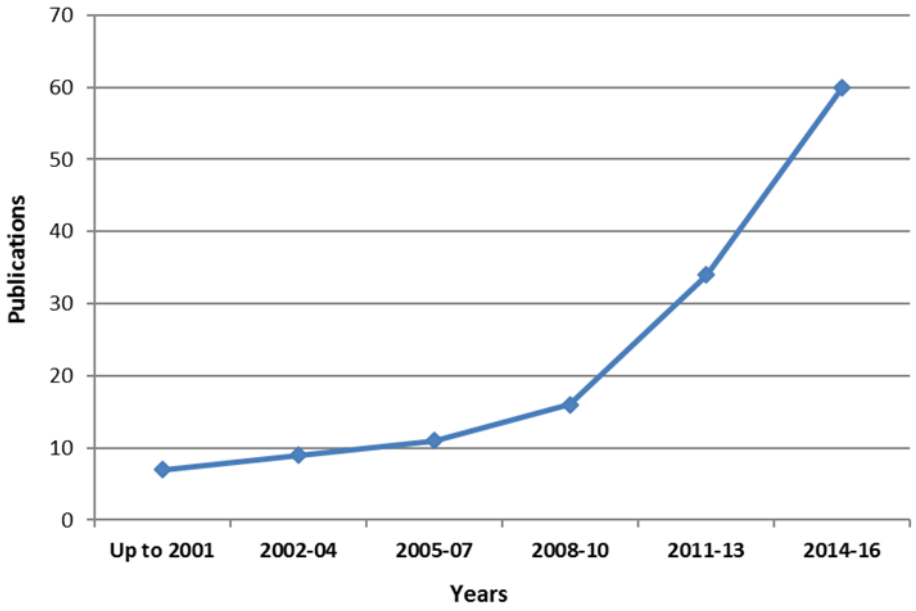


Figure 9. Temporal evolution of honey authentication research

Figure 10 shows that Mediterranean countries such as Italy, Spain, France and Greece, are scientifically active on the field. This is normal as these countries have both PDO and PGI honeys alongside with plenty of other authentic foodstuffs and wines. Furthermore, it has to be cited that countries such as the USA, a country with high scientific capabilities, remain back in the studies concerning honey authenticity. China shows also an intense interest pointing out the adulteration stories that have emerged in the country. Another important finding is that except USA and China all the other countries, in the top 10, are European

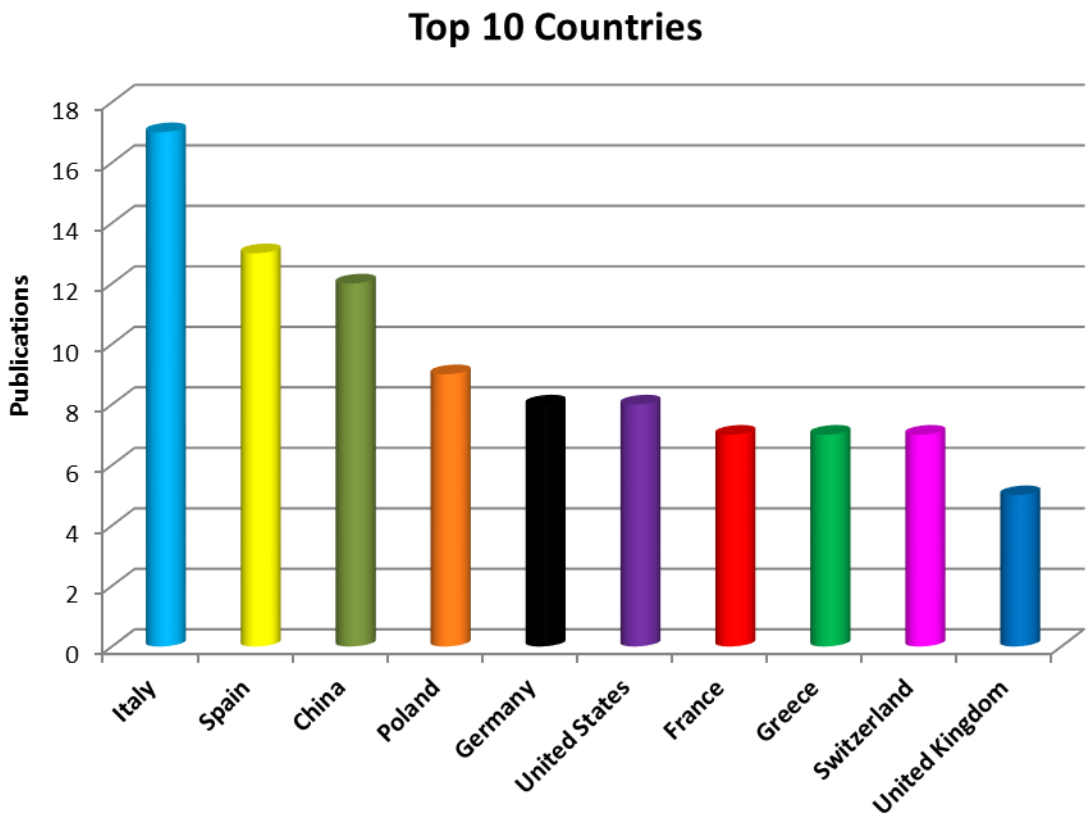


Figure 10. Top 10 countries on honey authenticity research

Publications on honey authentication are scattered in more than 50 journals. Consequently, honey authenticity is a multidisciplinary task. In figure 11 the top ten journals on the field are presented. However, there is a certain trend as the majority of scientists prefer to public their efforts on “Food Chemistry” (29%) and “Journal of Agricultural and Food Chemistry” (24%). These two journals are the most highly cited food journals. This indicates the importance of food authentication studies. The fact that food authentication is strongly based on analytical methodologies is shown by the presence of “pure” Analytical Chemistry journals, such as “Journal of Chromatography A”, “Talanta” and “Trends in

Analytical Chemistry". This indicates the need for reliable and robust new analytical methods for the verification of honey authenticity.

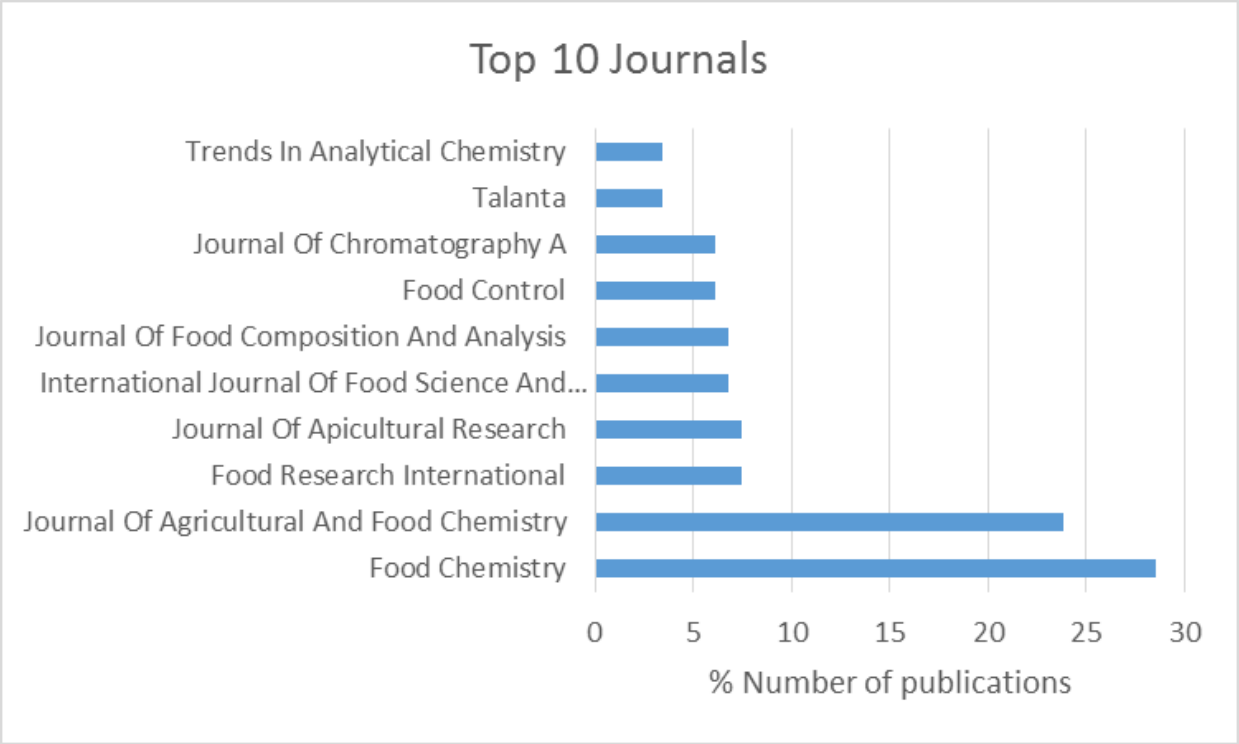


Figure 11. Top 10 journals on honey authenticity research

2.3 Characterization of honey as PDO, PGI, TSG

The standardization of food composition and processing is a practice that have started since the beginning of 20th century with the foundation of several organizations. Typical examples of that in Europe are the French "Institut National des Appellations d'Origine" (INAO), the Spanish "Denominación de Origen', the Italian "Denominazione di Origine Controllata", while in other parts of the world are the United States' "American Viticultural Areas" and South Africa's "Wine of Origin" [101].

In Europe, origin is one of the main authenticity issues. The legal framework for the GIs has comprehensively reviewed by Danezis et. al [98]. Briefly, the production of the member states was firstly protected in 1992 in respect of legislation. The European Economic Community (EEC, since 1993 European Union, EU) introduced the quality terms "protected designation of origin" (PDO)

and “protected geographical indication” (PGI), enacted with Council Regulation (EEC) No. 2081/92 [102] and Council Regulation 2082/92 [103]. In 2006, following the third reform of the Common Agricultural Policy (CAP) of 2003, the EU replaced Council Regulation (EEC) No. 2081/92 by Council Regulation (EC) No. 510/2006 [104]. At the beginning of 2013, Regulation (EC) No. 510/2006 was repelled by Regulation (EU) No. 1151/2012, the main legislation act that governs quality schemes in EU [105].

According to the Regulation (EU) No. 1151/2012 the products are indicated as

- “Protected Designation of Origin” – P.D.O (under Article 5 paragraph 1)
    - originating in a specific place, region or, in exceptional cases, a country
    - whose quality or characteristics are essentially or exclusively due to a particular geographical environment with its inherent natural and human factors
    - the production steps of which all take place in the defined geographical area.
- “Protected Geographical Indication” – P.G.I (under Article 5 paragraph 2)
    - originating in a specific place, region or country
    - whose given quality, reputation or other characteristic is essentially attributable to its geographical origin
    - at least one of the production steps of which take place in the defined geographical area.
- “Traditional Specialities Guaranteed” – T.S.G (under Article 18 paragraph 1)
    - results from a mode of production, processing or composition corresponding to traditional practice for that product or foodstuff
    - is produced from raw materials or ingredients that are those traditionally used.

According to the commission Implementing Regulation (EU) No 668/2014, which laid down the rules for the application of Regulation (EU) No 1151/2012, special signs are enacted for these quality schemes. Figure 12 depicts different versions of the EU official logos for the existing categories of geographical indications.

Figure 12. Official signs of the PDO, PGI and TSG products

To date (4/2017), in the EU there are 671 PDOs, 816 PGIs, and 80 TSGs registered products as presented at DOOR, the official database for registration of food products of the European Commission. PDOs and PGIs have proven a great potential increasing the value of the registered foodstuffs. On the other hand, TSG scheme has not been that successful compared to the other quality schemes because of legislative structure difficulties [106].

The Regulation (EU) No. 1151/2012 also introduced the concept of “optional quality terms” importing two new categories. These optional quality schemes were enacted in order to increase the value of rural areas, where inhabitants face difficulties in order to cultivate and produce their products. A product is identified as

- Mountain product (under Article 31 paragraph 1)
    - both the raw materials and the feedstuffs for farm animals come essentially from mountain areas
    - in the case of processed products, the processing also takes place in mountain areas.
- Product of Island farming (under Article 32 paragraph 1)

- materials of which come from islands.
- in the case of processed products, processing must also take place on islands.

Honey belongs to class 1.4 of Commission DOOR database, which also includes other product of animal origin such as eggs, various dairy products except butter, etc. In order to include a particular product under the PDO classification, the producer should send an application for registration on PDO system. The application should contain product specification and all the technical information necessary to describe the product. The specification is the main evidence to support the PDO application. The product specification shall include at least:

- the name of the agricultural product
- the proposed quality scheme
- description of the product
- physical, chemical, microbiological, organoleptic characteristics
- the geographical area
- production process
- inspection structures
- labelling details

In the case of honey, the description of the product features the following characteristics [107]. Quality criteria as water content, HMF, conductivity, color, sugars content etc. Additionally, pollen characteristics are always required. Method of production also is presented. Specification of honey remove and extraction method, packaging, and storage are important because they are essential in maintaining the characteristics and value of product. The link with the geographical area is an essential requirement in the case of PDO, and it must demonstrate why honey is associated with one particular area and not another. Historical data, soil, climate condition, agricultural activity, and other environmental factors, and especially its flora, are causal link between the geographical area and the quality of honey. At present, 35 PDO and PGI honeys (27 PDO and 8 PGI) have been registered and applied. Portugal is the leading country (9 products)

followed by Spain and France (7 and 5 products respectively). Finally, Croatia, Greece, Italy, Lithuania, Luxembourg, Poland and Slovenia are the rest of the countries figure 13.

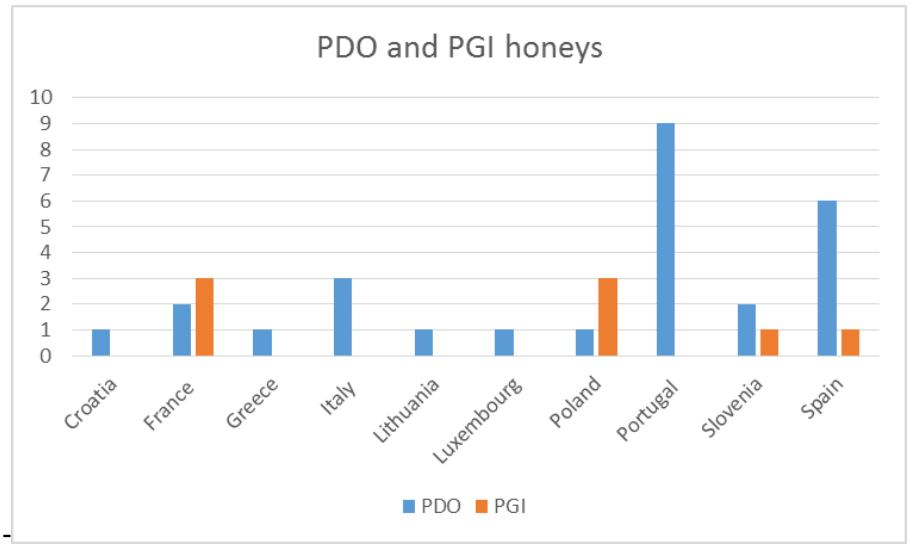


Figure 13. PDO and PGI honeys (class 1.4) as obtained by Commission DOOR database, 4/2017

2.4 Techniques used in honey authenticity studies

Determining the authenticity could involve a range of verification approaches depending on the level of sophistication of the fraud itself. This section provides insight on analytical techniques needed to verify the origin of honey, in terms of regulatory and more recently consumer and industry requirements. Various analytical techniques have been assessed on their suitability for food authentication studies throughout the years. As shown in figure 14, chromatography and fluorescence and vibrational spectroscopy are the 2 major groups of techniques which are mostly used. Almost half of the research articles are in these two groups. Thereafter, isotopic, NMR and other techniques like those which determine physicochemical characteristics of honey e.g. electrical conductivity are following. Some other concepts that should not be ignored are elemental techniques and sensory analysis. All the aforementioned techniques provide a vast amount of analytical data. The information which is included in this

huge amount of data can only be extracted using chemometric tools. Multianalyte capabilities are essential for food authentication studies as more descriptors facilitate better classification. To this end, various techniques described either under the term chemometrics or data mining are crucial for successful prediction

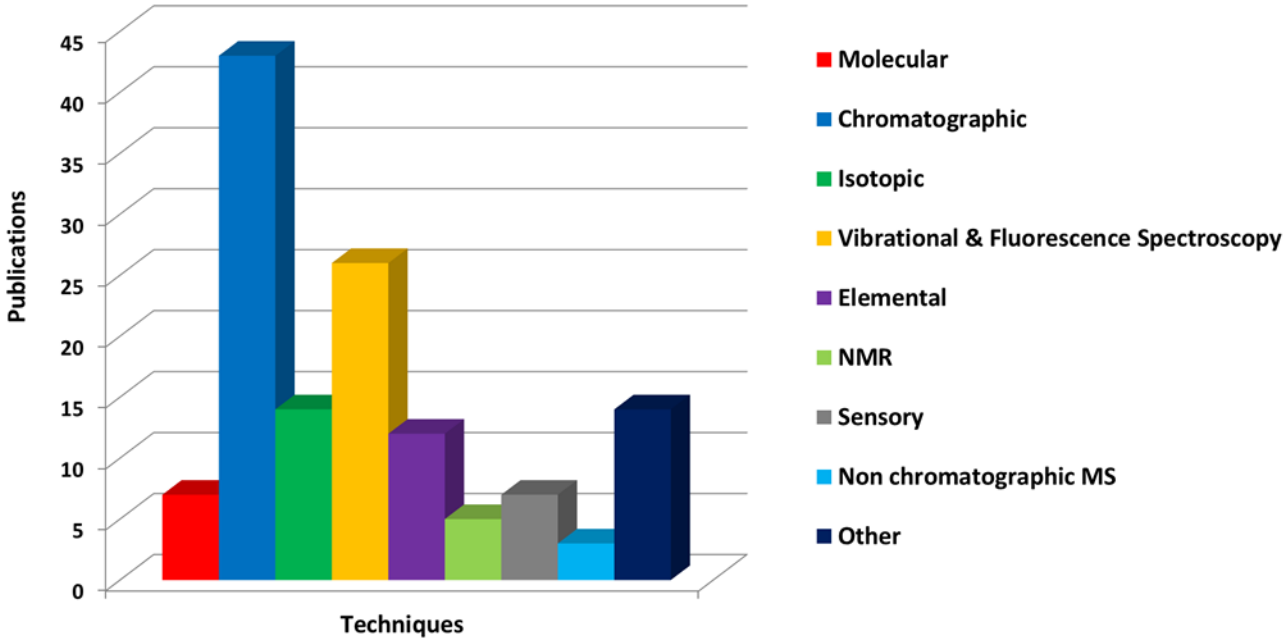


Figure 14. Analytical techniques used in honey authenticity studies

models and utilization of the data.

2.5 Authenticity and discrimination markers in honey authenticity studies

The great variety of honey compounds originate from the melliferous flora and the honeybee processing on collected nectar and honeydew. In this way, the presence and quantity of the various organic and inorganic constituents is strictly associated to the botanical origin. However, plants may originate in various geographical areas resulting to differences in composition. In fact, the combination of botanical origin with soil and climate characteristics, reflects the holistic impact of origination on composition. Floral, regional, territorial or topographical origin should be specified in order to produce an authentic product. Potential authenticity markers and discrimination patterns are widely cited in the literature. These markers include, but are not limited to, phenolic compounds, isotopic profile, mineral content and physicochemical characteristics. Moreover, the determination of aforementioned analytes is carried out using various analytical features, as already mentioned in the previous paragraph. Table 4 indicatively summarizes the various authenticity markers alongside the scope of each study, the pretreatment of the samples and the technique used.

Table 4. Authenticity markers for the discrimination and verification of geographical and botanical origin

Authenticity issue	Sample preparation	Compounds of interest/ Best markers - indicators	Technique	Ref
Geographical origin & botanical origin	Acid extraction, filtration & SPE cartridge	Dicaffeoylquinic, ellagic, caffeic and chlorogenic acids, quercetin, bis-methylated-quercetin, methoxychrysin, pinocembrin, isorhamnetin, pinobanksin, galangin, sacuranetin, acacetin, eriodictyol, phenethyl ester and 5-methylether-3-O-acetate.	UPLC - orbitrap MS	[108]
Geographical and botanical origin, adulteration with glucose and saccharose syrups	Dilution	Different responses using Pt, Au, Pd, Cu, GC, Ni and Ag electrodes	Voltammetric electronic tongue	[109]

Geographical and botanical origin	lyophilization process, appropriate dilution, ADCS with Cadiot columns for ethanol & water extraction	$\delta^{13}C$, $\delta^{18}O$ and $\delta^{2}H$ along with $(D/H)_I$ from ethanol	IRMS and SNIF-NMR	[110]
Geographical and botanical origin	liquefied at 55 °C for 8 h, allowed to cool to room temperature	Fluorophores compounds such as polyphenols and amino acids.	Front-Face Fluorescence Spectroscopy	[111]
Botanical origin	Solid phase extraction (SPE), using C18 cartridges	caffeic acid, chlorogenic acid, ellagic acid, p-coumaric acid, quercetin and hesperetin	HPLC-UV	[112]
Botanical origin	Acid extraction & filtration	quercetin, myricetin and luteolin	HPLC-UV	[113]
Botanical origin	Acid extraction & filtration	gallic, abscisic, ellagic and coumaric acids	HPLC-UV	[114]
Botanical origin	Acid extraction & filtration	Naringenin, Abscisic acid [(trans, trans) & (cis, trans)]	HPLC-DAD	[115]
Botanical origin	fiber DVB-CAR-PDMS, dilution, heating	hotrienol and β-pinene	SPME GC-MS	[116]

Botanical origin	-	pH, total acidity solutions, specific electrical conductivity, dynamic viscosity, diastatic number, 5-HMF and proline content	physicochemical parameters	[117]
Botanical origin	Dilution - centrifugation	Melissopalynological analysis (pollen analysis) Pollens: R. pseudoacacia, Citrus spp., Castanea,	Optical microscope & FT-Raman spectroscopy	[118]
Botanical origin	Acid digestion	Fe, Mn, Zn, Cu and Hg	ICP-MS	[119]
Botanical origin	Microwave acid digestion	Na, Mg, K, Ca, Mn, Fe, Cu, Rb, Sr and Ba	ICP-MS	[120]

CHAPTER 3
Scope & Objectives

Honey is a foodstuff which is subjected to various frauds like addition of syrups or mislabeling due to its high price in the market. Particularly, misdiscreption of unifloral honeys is a common practice as the production of this type of honey is a laborious procedure. Moreover, the honey obtained by specific plants is strictly connected with unique organoleptic or health beneficial properties. Another important factor influencing the final value of the product is the provenance in which it is produced. On this merit, the evaluation and verification of honey authenticity is a task of great importance for the producers, consumers and regulatory bodies.

Analytical chemistry plays a decisive role in the fight against the mislabeling of honey. Various analytical methods for the determination of botanical and geographical origin have been developed based on chromatographic, isotopic, elemental and spectroscopic principles. Accordingly, a great variety of analytes has been shown potential as origin markers. Phenolic compounds have been chosen as the analytes of this study because of their proven capability to differentiate the origin of honey and their beneficial properties on human health.

So, the aim of this master thesis is the evaluation of the phenolic content of various unifloral honeys and the potential discrimination of the botanical and geographical origin based on the polyphenols content. Particularly, the objectives of this study are:

- the development and validation of a UPLC-QToF MS method for the determination of the phenolic compounds
- the target screening of 22 polyphenols in 9 unifloral honeys

- the establishment of a suspect list including 64 compounds that have been already detected in previous studies and the suspect screening of these compounds in some of the samples

Concluding, in the end of the present master thesis, future perspectives and work to be done are discussed.

CHAPTER 4

Materials and methods

4.1 Reagents, standards and solvents

For the UHPLC-ESI-QToF system:

- Methanol (MeOH hypergrade for LC-MS, Sigma-Aldrich)
- Ultrapure water (18.2 MΩ cm^{-1}, produced by a Milli-Q water purification system)
- Acetonitrile (ACN LC-MS grade, Merck)
- Ammonium acetate (Fluka, Sigma-Aldrich).
- Sodium formate (Sigma Aldrich)

For the experimental procedure:

- Syringic acid (purity 95 %), myricetin (purity 99%), eriodictyol (purity 99%) and taxifolin (purity 99%) were purchased from Extrasynthèse (Genay, France), 4-hydroxybenzoic acid (purity 99%), 3,4-dihydroxybenzoic acid (purity 97%), 2,5-dihydroxybenzoic acid (purity 99%), salicylic acid (purity 99%), vanillic acid (purity 97%), gallic acid (purity 98 %), ferulic acid (purity 98 %), epicatechin (purity 97 %), p-coumaric acid (4-hydroxycinnamic acid; purity 98 %), quercetin (purity 98 %) were obtained from Sigma-Aldrich (Steinheim, Germany), hydroxytyrosol (purity 98 %) and luteolin (purity 98 % was purchased from Santa Cruz Biotechnologies, caffeic acid (purity 99 %), vanillin (purity 99 %), ethyl vanillin (purity 98 %), apigenin (4,5,7-trihydroxyflavone; purity 97 %), and tyrosol [2-(4-hydroxyphenyl) ethanol, purity 98 %] were acquired from Alfa Aesar (Karlsruhe, Germany), cinnamic acid (purity 99%) was purchased from Merck (Hohenbrunn, Germany).
- Stock solutions of 1000 mg L^{-1} were prepared for each analyte. 0.01g was weighed and diluted in MeOH in 10 mL volumetric flask. The solutions were stored at −20 °C in amber glass bottles to prevent photodegradation.
- Working solution of 25 mg L^{-1} was prepared. The working solution contained all the analytes. 40 µl of each analyte from stock solutions were transferred and diluted in MeOH in a 25 ml volumetric flask. The solution was stored at −20 °C in amber glass bottle to prevent photodegradation.
- Ethyl Acetate (Sigma Aldrich)
- Sodium sulfate (Sigma Aldrich)
- Sodium chloride (Carlo Ebra reagents)

- Magnesium sulfate (Sigma Aldrich)

All the necessary dilutions were performed to standards and stock solutions according to the experimental requirements, in order to prepare the diluted standards. All working solutions were stored in the refrigerator.

4.2 Sampling and storage

51 honey samples were collected from Greek and Polish producers. All samples were characterized as organic and conventional, or according to their botanical type, based on their certified labeling. Samples were shelled and preserved in a dark and cold room before analysis. Details considering geographical origin, botanical type and production type of honey samples are presented in Table 5.

Table 5. Characterization of honey samples

sample ID	Botanical origin	Production type	Geographical origin
r3	acacia	Conventional	POLISH
r19	acacia	Organic	POLISH
r23	acacia	Organic	POLISH
r30	acacia	Conventional	POLISH
r35	acacia	Conventional	POLISH
r54	acacia	Conventional	POLISH
b1	arbutus	Organic	GREEK
b17	arbutus	Organic	GREEK
b14	blossom	Organic	GREEK
b22	blossom	Conventional	GREEK
r5	buckwheat	Conventional	POLISH

r8	buckwheat	Organic	POLISH
r14	buckwheat	Organic	POLISH
r25	buckwheat	Organic	POLISH
r33	buckwheat	Conventional	POLISH
r39	buckwheat	Conventional	POLISH
r45	buckwheat	Conventional	POLISH
-r57	buckwheat	Conventional	POLISH
b16	chestnut	Organic	GREEK
b21	chestnut	Conventional	GREEK
b8	fir	Organic	GREEK
b27	fir	Organic	GREEK
r27	heather	Conventional	POLISH
r28	heather	Conventional	POLISH
r29	heather	Conventional	POLISH
r31	heather	Conventional	POLISH
r37	heather	Conventional	POLISH
r46	heather	Conventional	POLISH
r60	heather	Conventional	POLISH
b12	heather	Organic	GREEK
b25	heather	Organic	GREEK
b28	heather	Conventional	GREEK

r6	linden	Conventional	POLISH
r9	linden	Organic	POLISH
r12	linden	Organic	POLISH
r13	linden	Organic	POLISH
r42	linden	Conventional	POLISH
r47	linden	Conventional	POLISH
r48	linden	Conventional	POLISH
r49	linden	Conventional	POLISH
r59	linden	Conventional	POLISH
r20	rape	Organic	POLISH
r26	rape	Conventional	POLISH
r40	rape	Conventional	POLISH
r50	rape	Conventional	POLISH
r51	rape	Conventional	POLISH
r52	rape	Conventional	POLISH
r53	rape	Conventional	POLISH
b2	thyme	Organic	GREEK
b13	thyme	Organic	GREEK
b18	thyme	Organic	GREEK

4.3 Sample preparation

1 g of homogenised honey was diluted with 5 mL of acidified water (pH<2) with the addition of 2% sodium chloride. After being vortexed for 1 min, the diluted honey

was extracted 3 times with 5 mL ethyl acetate (EtAc), respectively. Between each extraction the samples were centrifuged in order to obtain a better separation of the two phases. The combined organic phases were dried with sodium sulphate. Extracts were evaporated under a gentle nitrogen stream near to dryness and then reconstituted to 0.2 mL with a final proportion of MeOH:H2O (50:50). Finally, the extracts were filtered through a 0.2 µm RC syringe filter and were ready for injection in the RP chromatographic system.

During the development of the method acetonitrile and a solution of acetonitrile: ethyl acetate (50:50) were also tested. The protocol for these trials was exactly the same. In the case of acetonitrile magnesium sulfate was also used for a better separation between the water and the organic phase.

4.4 UHPLC-HRMS/MS system and analysis

Honey samples analysis was carried out using an UHPLC-QToF-MS system composed of:

- An UHPLC rapid separation pump system, Dionex UltiMate 3000 (Thermo Fisher Scientific)
- Autosampler
- QToF mass spectrometer, Maxis Impact (Bruker Daltonics)

Mass spectra acquisition and data analysis was processed with DataAnalysis 4.3 and TargetAnalysis 1.3 (Bruker Daltonics, Bremen, Germany). The QToF-MS system is equipped with an ESI source, operating in negative ionization mode. The chromatographic separation was performed on reversed-phase (RP) chromatographic system.

In RP mode, an Acclaim RSLC C18 column (2.1 × 100 mm, 2.2 µm) from Thermo Fisher Scientific, connected to an ACQUITY UPLC BEH C18 1.7 µm, VanGuard Pre-Column from Waters, and thermostated at 30 °C, was used.

Figure 15. The UHPLC-QToF-MS system

For negative ionization mode, the aqueous phase consisted of H_2O:MeOH 90:10 with 5 mM ammonium acetate and the organic phase comprised of MeOH with 5 mM ammonium acetate.

The elution gradient program started with 1% of organic phase (flow rate 0.2 mL min^{-1}) for one minute, increasing to 39 % by 3 min (flow rate 0.2 mL min^{-1}), and then to 99.9 % (flow rate 0.4 mL min^{-1}) in the following 11 min. These almost pure organic conditions were kept constant for 2 min (flow rate 0.48 mL min^{-1}) and then initial conditions were restored within 0.1 min, kept for 3 min and then the flow rate decreased to 0.2 mL min^{-1} for the last minute. The injection volume was set to 5 µL.

When RP chromatographic system was used, the operation parameters of ESI were the following: capillary voltage, 2500 V for positive and 3000 V for negative mode; end plate offset, 500 V; nebulizer pressure, 2 bar (N_2); drying gas, 8 L min−1 (N_2); and drying temperature, 200 °C

All the samples were first analyzed in full scan mode. The QTOF-MS system was operating in broadband collision-induced dissociation (bbCID) acquisition mode and recorded spectra over the range m/z 50−1000 with a scan rate of 2 Hz. The Bruker bbCID mode provides MS and MS/MS spectra at the same time working at two different collision energies; at low collision energy (4 eV), MS spectra were acquired, where all of the ions from the preselected mass range are heading towards the flight tube without isolation at the quadrupole and there is no collision-induced dissociation at the collision cell. At high collision energy (25 eV), no isolation is taking place at the quadrupole, and the ions from the preselected mass range are fragmented at the collision cell.

For certain masses of interest, a second analysis including the list of the selected precursor ions was performed in AutoMS (data dependent acquisition) mode. The instrument provided a typical resolving power (full width at half maximum) between 36,000 and 40,000 at m/z 226.1593, 430.9137, and 702.8636.

A QTOF-MS external calibration was daily performed with a sodium formate solution, and a segment (0.1−0.25 min) in every chromatogram was used for internal calibration, using a calibrant injection at the beginning of each run. The sodium formate calibration mixture consists of 10 mM sodium formate in a mixture of water/isopropanol (1:1). The theoretical exact masses of calibration ions with formulas $HCOO(NaCOOH)_{1\text{-}14}$ in the range of 50−1000 Da were used for calibration.

CHAPTER 5
Results and discussion

5.1 Development and validation of the method

The development and validation of a method is a crucial analytical challenge. In this stage, all analytical parameters such as sample pretreatment, mobile phase composition and flow as well as MS parameters have to be optimized in order to achieve the best results. In this study, the extractant is the only parameter that has been examined using the recovery rate of the phenolic compounds as criterion for the evaluation of the extraction efficiency. The main reason for this choice is that a previously optimized LC-MS method is used because many chemometric models such as in-house retention time prediction model are developed [121]. So, only in this way these tools can be utilized and proceed with non-target screening so as to obtain discrimination patterns for the verification of the samples authenticity. Regarding to the validation part, it is also an important analytical issue as it demonstrates if the developed method is fit for purpose. It is important to notice that although nowadays the attention for the presence of basic validation data in scientific publications is surely higher than in past years, unfortunately more than 30% of the methods reported in literature are completely not-validated [21]. This fact raises some doubts on the non-validated results reliability as validation represents an essential component of the measures that any laboratory should implement to produce reliable analytical data. The results of the recovery rate using different extraction solvents and the validation process are presented and discussed in the following pages.

5.2 Selection of the extraction solvents

The highest extraction efficiency towards the target phenolic compounds was achieved by testing 2 different solvents and a mixture of them. Namely, ethyl acetate (100%), acetonitrile (100%), and EtAc:ACN (50:50), were assayed and

evaluated based on the recovery rate. The compounds were spiked from the 25 ppm standard mix solution in order to have a final concentration of 1 ppm. In detail, the mix was composed of: apigenin, ethyl vanillin, ferulic acid, gallic acid, hydroxytyrosol, luteolin, p-coumaric acid, quercetin, tyrosol, vanillin. It has to be noted that only these standards were available during the validation process. Three subsamples were extracted with each extractant and analyzed. The results from this testing experiment are presented in table 6.

Recovery rate was measured using the following equation

$$R\% = \frac{Area\ spiked - Area\ sample}{Area\ matrix\ matched - Area\ sample}\ x\ 100 \qquad (1)$$

Table 6. Recovery rate % for each spiked compound in the three different extractants

	Extractant (n=3)		
compound	**ethyl acetate**	**acetonitrile**	**ethyl acetate : acetonitrile, 1:1**
Apigenin	117	112	50
Ethyl vanillin	105	93	51
Ferulic acid	121	68	71
Hydroxytyrosol	105	102	74
Luteolin	97	95	50
p-coumaric acid	101	48	66
Quercetin	119	103	33
Tyrosol	111	110	75
Vanillin	97	81	50

To begin with, the mixture of EtAc and ACN (50:50) proved to be inefficient for the extraction of flavonoids and phenolic compounds. A recovery rate ranged from 30 to 75% was observed. On the other hand, satisfactory recovery rates were achieved using EtAc and ACN as extractant as they ranged above 95% and 80%

respectively. However, the extraction efficiency for phenolic acids like ferulic and p-coumaric acid are significantly better utilizing EtAc as extractant. Recovery rates of 121% and 101% were obtained for ferulic and p-coumaric acid in the case of EtAc, while 68% and 48% using ACN. Phenolic acids are an important fraction of the polyphenols and as such they may significantly contribute to the aim of differentiation of samples according to geographical and botanical origin [122]. Additionally, as it has already described in previous section ethyl acetate is a common choice for the extraction of polyphenols in honey. Consequently, EtAc was chosen as the extractant of the developed method.

5.3 Method Validation

To demonstrate the feasibility of the present approach for the determination of phenolic compounds and to test its practicability, the performance of the method was fully evaluated in terms of, linearity, limits of detection (LODs) and limits of quantification (LOQs), repeatability (precision) and trueness. Oak honey samples were fortified with a mixture of the analytes. This mixture was composed by: apigenin, cinnamic acid, eriodictyol, ethyl vanillin, ferulic acid, hydroxytyrosol, luteolin, p-coumaric acid, quercetin, taxifolin, tyrosol, vanillin, vanillic acid. It has to be noticed that the great majority of the analytes used in the validation process have been cited before in literature to be existed in honey samples. Spiking was performed in 6-fold at three levels (0.5, 1 and 2 mg Kg^{-1}) and 3-fold at two levels (0.25 and 5 mg Kg^{-1}). Together with control samples, the fortified samples were processed using the extraction procedure described above. Aliquots of the control extracts were used to prepare matrix matched calibration standards at 0.25, 0,5, 1, 2, 5 mg/Kg. The same calibration standards were also prepared in methanol: water (50:50).

At this point is should be highlighted that, the selection of the analytes are representative to their physiochemical properties. Flavonoids like hydroxytyrosol or tyrosol will not occur in honey. However as to study the methods flexibility regarding its scope, some of the combinations analyte/matrix are used as indicators of the analytical behavior of a larger group of molecules with similar

physicochemical properties in the complex honey matrix. These knowledges can be extrapolated in the future for expansion of the scope of the method to new foodstuffs in which some of the analytes will be presented.

The results of validation are summarized in table 7 and are discussed in the following pages. Recoveries and ME% values at the level of 2 mg Kg^{-1} are presented in the table 7.

Table 7. Results of the validation of the target screening method

Compound	LOD mg / Kg	LOQ mg / Kg	Repeatability, RSDr %, (n=6)	Equation	r^2	Recovery %	ME %
Apigenin	0.15	0.45	1.1	y = 562997x + 134183	0.990	80	8
Cinnamic acid	0.029	0.086	2.5	y = 66165x - 4335.9	0.999	72	-21
Eriodictyol	0.039	0.12	11	y = 15507x - 654.54	0.999	91	27
Ethyl vanillin	0.027	0.081	2.2	y = 113199x + 161.93	0.999	68	-4.0
Ferulic acid	0.083	0.25	4.3	y = 36113x + 5878.6	0.992	91	-14
Hydroxytyrosol	0.020	0.060	2.3	y = 60544x + 1039.7	0.999	73	1.0
Luteolin	0.17	0.51	1.6	y = 495013x + 4237.7	0.999	98	17
p-coumaric acid	0.071	0.21	6.1	y = 25464x + 2671.3	0.994	76	6.0
quercetin	0.025	0.074	0.89	y = 300727x + 6175.7	0.993	87	-18
taxifolin	0.034	0.10	18	y = 141285x + 10062	0.998	83	26
Tyrosol	0.019	0.057	4.4	y = 34426x - 975.82	0.999	80	-22
Vanillin	0.042	0.12	3.7	y = 81019x - 8526	0.998	70	-16
vanillic acid	0.058	0.17	3.1	y = 16207x + 6942	0.996	73	-11

5.3.1 Linearity

Good linearity was achieved in most cases with correlation coefficients better than 0.99 in 79% of the cases. Only apigenin, epicatechin and luteolin had correlation coefficient about 0.97. For these three compounds, when the highest concentration level (5 ppm) was excluded a correlation coefficient of >0.99 was achieved. According to the standard solutions prepared in methanol:water (50:50) only apigenin had r^2<0.99. In figure 16, the calibration curve of p-coumaric acid is indicatively presented.

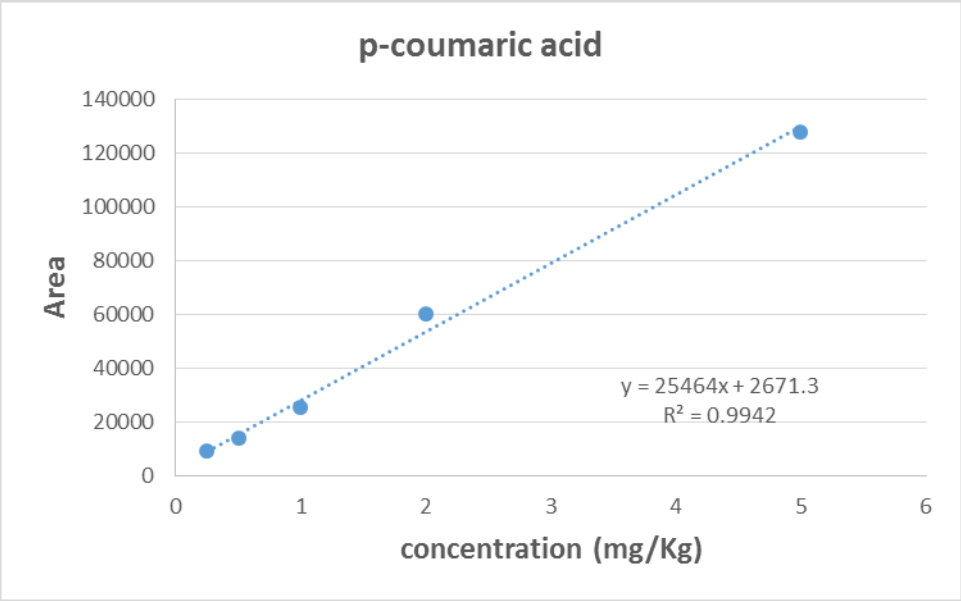


Figure 16. Calibration curve of p-coumaric acid

5.3.2 LODs and LOQs

LODs and LOQs were calculated from the matrix-matched calibration curves based on the following equations,

$$LOD = \frac{SDintercept}{slope}\ x\ 3.3 \qquad (2)$$

$$LOQ = \frac{SDintercept}{slope}\ x\ 10 \qquad (3)$$

LODs and LOQs were very good and the method is proved to be suitable for the detection of phenolic compounds in low concentration. The values for LODs ranged between 0.019 mg Kg^{-1} (tyrosol) and 0.17 mg Kg^{-1} (luteolin) while for LOQs ranged between 0.057 and 0.51 mg Kg^{-1} respectively.

5.3.3 Precision – RSDr %

Precision was evaluated for each phenolic compound by spiking all analytes in three different concentration levels (0.5 , 1, 2 mg Kg^{-1}) in terms of the relative standard deviation (RSD_r %). A total of six replicates in each level was performed. Figure 17 shows an overview of the RSD_r % for the various analytes. The vast majority of the analytes showed RSD_r < 5% indicating the good precision of the developed method.

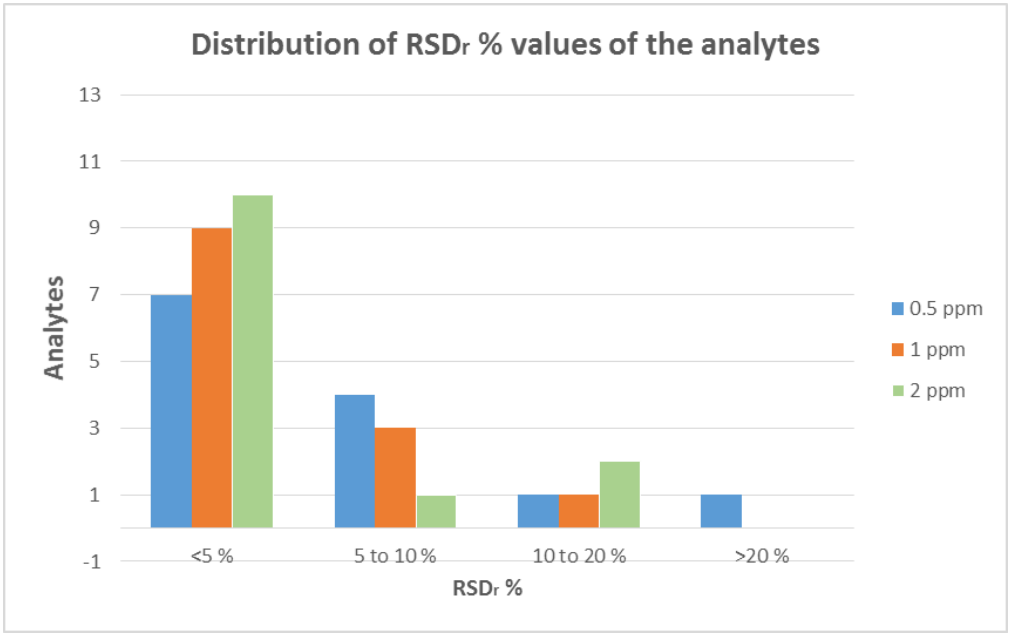


Figure 17. Distribution of the RSDr % values of the analytes at 3 different levels

5.3.4 Matrix Effect % (ME %)

The measurement of matrix effect (ME) is an important issue as the selectivity of a proposed method is investigated. According to Truffeli et. al. [123] ion suppression or enhancement may be caused by sample matrix or interferences from metabolites. The mechanism and the origin of the matrix effect is not fully understood, but it may originate from the competition between an analyte and a

co-eluting compound or undetected matrix components reacting with primary ions formed in the interface. Depending on the environment in which the ionization and ion evaporation processes take place, this competition may effectively decrease (ion suppression) or increase (ion enhancement) the efficiency of formation of the desired analyte ions present at the same concentrations in the interface. It is intuitively clear that the efficiency of formation of the desired ions is matrix-dependent due to the competition between the molecule of interest and a number of other undetected but co-eluting molecules present in the system that are capable of reacting with primary ions. This effect may reduce or increase the intensity of analyte ions and affect the reproducibility and accuracy of the assay.

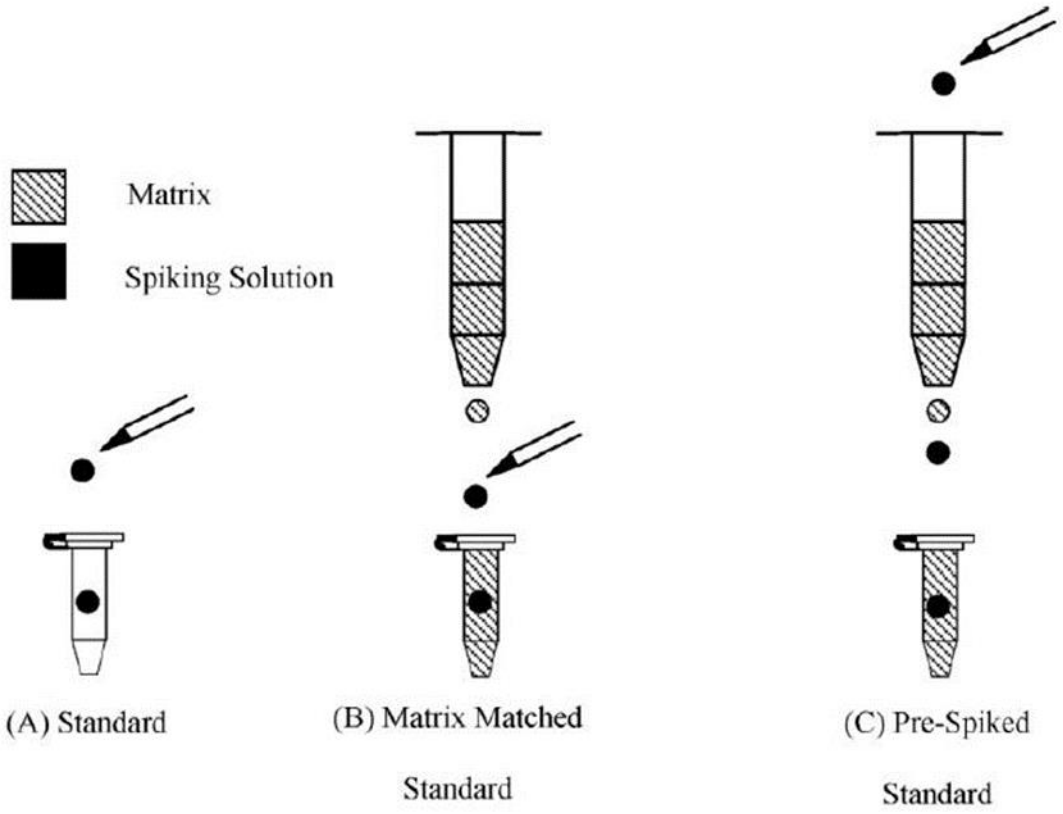


Figure 18. Preparation of solutions for the determination of recovery rate and matrix effect, source : [123]

In order to measure the ME, the matrix factor (MF) is necessary. MF and ME are calculated based on the following equations

$$MF = \frac{Area\ matrix\ matched - Area\ sample}{Area\ standard - Area\ sample} \quad (4)$$

$$ME\ \% = (1 - MF)\ x\ 100 \quad (5)$$

Ideally, the ME % is equal to zero meaning that neither ion suppression nor enhancement is taken place. It has to be noticed that positive values of ME% indicate ion suppression while negative enhancement. According to the ME% values of the developed method values between -20% to 20% were found. The signal of phenolic acids like cinnamic (ME= -21%) and ferulic (ME= -14%) acid was slightly enhanced, while these of flavonoids like apigenin (ME= 8%) and luteolin (ME= 17%) was slightly suppressed.

5.3.5 Trueness

Accuracy is one of the key parameters to be assessed for method validation and involves common systematic errors (bias). It is estimated through trueness and precision. Trueness is usually estimated using certified reference materials (CRM), but in cases where this is not feasible measurements through recovery of additions of known amounts of the analytes to a sample (blank matrix) can be utilized. Recovery experiments were conducted at 5 spiking levels (0.25, 0.5, 1, 2, 5 mg Kg^{-1}) and in 3 replicates for each level. The % recoveries were calculated using the equation (1). The calculations were performed using matrix-matched calibration standards so as to take into account the matrix effect in quantitation. Generally, the majority of the analytes presented satisfactory recoveries ranging between 70% and 110%. Generally, mean recoveries of 70 -120% with relative standard deviations RSD <20% are acceptable when referring to validation experiments, while in certain cases. The method is still capable to serve a quantitative determination even if are lower than 70% or higher than 120%. In this case, the final concentration of the analyte in the sample has to be corrected with the recovery. Figure 19 shows an overview of the R% for the various analytes. The

vast majority of the analytes showed R% values between 70 and 100% indicating the accurate determination of the method developed.

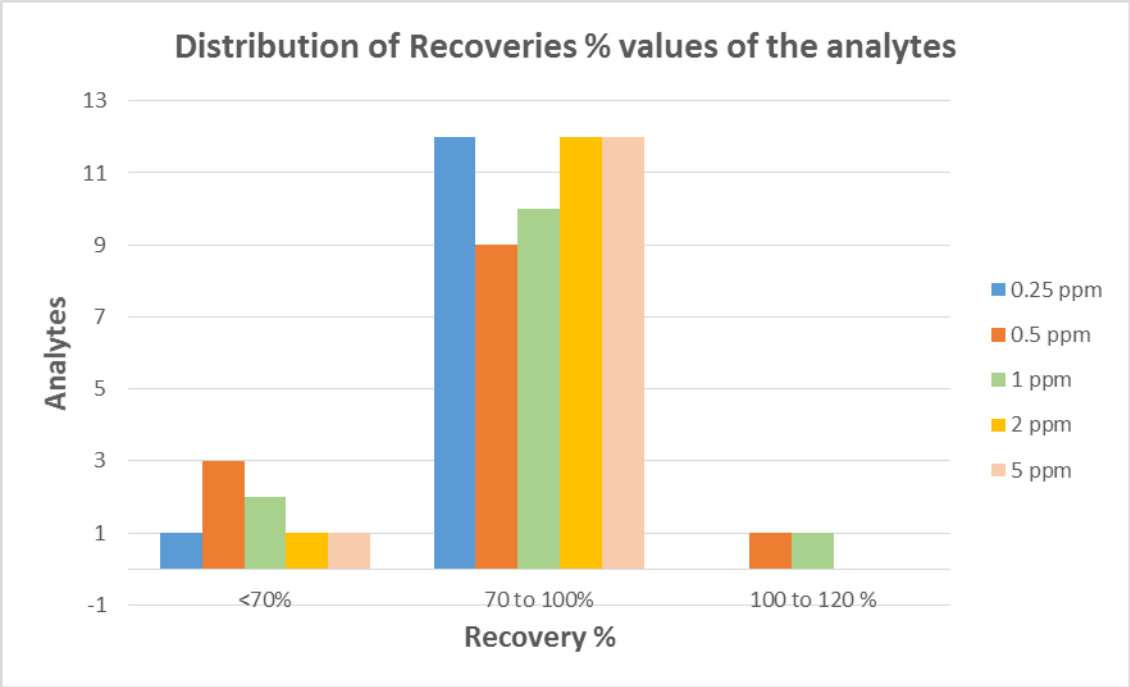


Figure 19. Distribution of the R % values of the analytes at 5 different levels

5.4 Target screening database

A data-dependent method was used to scan real honey samples for the presence of target compounds, and the presence of 22 target compounds was determined. The list comprises of both phenolic acids such as 3,4- dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, 4-hydroxybenzoic acid, cinnamic acid, salicylic acid, vanillic acid, ferulic acid, p-coumaric acid and polyphenols such as tyrosol and hydroxytyrosol from the phenolic alcohols, vanillin and ethyl vanillin from the phenolic aldehydes, apigenin, eriodictyol, quercetin, taxifolin and myricetin from the flavonoids, pinoresinol, which is a lignan. The mass accuracy of the precursor ions as well as those of the qualifiers of the detected compounds were less than 2 mDa compared with standard solutions and the isotopic fit was less than 50 mSigma in all cases.

The most abundant fragments provided by the MS/MS (AutoMS) spectra were confirmed by comparing with MassBank (www.massbank.eu) and literature records. Target screening results are summarized in table 8. The MS/MS fragments of the target compounds are presented in table 8.

Our results came in accordance with previous studies in which the MS/MS spectrum of phenolic compounds have been investigated [18, 108]. For instance, the MS/MS spectrum of taxifolin shows a fragment at m/z 285.0408, corresponding to $C_{15}H_9O_6$. Additionally, the qualifier ions of luteolin m/z 133.0287, corresponding to $C_8H_5O_2$, has also reported. p-coumaric acid shows characteristic fragmentation at m/z 119.0502 and 93.0344, corresponding to C_8H_7O and C_6H_5O respectively.

Table 8. Target screening results

Compound	Molecular formula	[M−H]− m/z standard	[M−H]− m/z experimental	tR (min)	q1 m/z	q1 formula	q2 m/z	q2 formula	q3 m/z	q3 formula
2,5-dihydroxybenzoic acid (gentistic acid)	C7H6O4	153.0187	153.0192	1.9	108.0215		109.0278			
3,4- dihydroxybenzoic acid (Protocatechuic acid)	C7H6O4	153.0188	153.0192	1.3	109.0294	C6H5O2	108.0218			
4-hydroxybenzoic acid	C7H6O3	137.0238	137.0245	1.4	93.0342	C6H5O	65.0398	C5H5		
Apigenin	C15H10O5	269.045	269.0457	8.2	269.0455	C8H5O	151.0031	C7H3O4	117.0340	C8H5O
Caffeic acid	C9H8O4	179.0344	179.0347	1.4	135.0453	C8H7O2	134.0346			
Cinnamic acid	C9H8O2	147.0446	147.0451	4.1	103.0553	C8H7	147.0446		146.8973	
Epicatechin	C15H14O6	289.0712	289.0712	4.3	123.0452	C7H7O2	151.0401	C8H7O3	137.0244	C7H5O3
Eriodictyol	C15H12O6	287.0555	287.0563	6.3	151.0038	C7H3O4	135.045	C8H7O2		
Ethyl vanillin	C9H10O3	165.0551	165.0554	5.4	136.0156		137.0222		108.0219	
Ferulic acid	C10H10O4	193.0501	193.0504	2.7	134.0361		178.026		149.061	
Gallic acid	C7H6O5	169.0137	169.0139	1.2	125.0244	C6H5O3	69.0344	C4H5O	97.0295	C5H5O2
Hydroxytyrosol	C8H10O3	153.0551	153.0553	3.5	123.0452	C7H7O2				
Luteolin	C15H10O6	285.0399	285.0398	7.4	133.0287	C8H5O2				
Myricetin	C15H10O8	317.0297	317.0301	6.2	151.0035	C7H3O4	178.9986	C8H3O5	317.0303	C15H9O8
p-coumaric acid	C9H8O3	163.0395	163.0395	2.5	119.0502	C8H7O	93.0344	C6H5O		
Quercetin	C15H10O7	301.0348	301.0358	7.1	151.0036	C7H3O4	178.9959	C8H3O5	121.0288	C7H5O2
salicylic acid	C7H6O3	137.0238	137.0242	3.2	93.0340	C6H5O	65.0399	C5H5		
Syringic acid	C9H10O5	197.045	197.0455	1.4	123.0080	C6H3O3	166.9976	C7H3O5		
Taxifolin	C15H12O7	303.0504	303.0511	4.7	125.0227	C6H5O3	285.0408	C15H9O6	153.0193	C7H5O4
Tyrosol	C8H10O2	137.0602	137.0609	4.1	119.0495		107.0496		93.034	

Vanillic acid	C8H8O4	167.0344	167.0349	1.4	125.0244	C6H5O3				
Vanillin	C8H8O3	151.0395	151.0399	4.6	136.0158					

The composition of the target screening database was performed as it described in this paragraph. The case of p-coumaric acid is used as example. Firstly, the EIC of the compound was extracted and MS and MS/MS were obtained. In this way, all the necessary information for the confirmation of the compound is available. The same procedure was followed for every phenolic compound existing in the database.

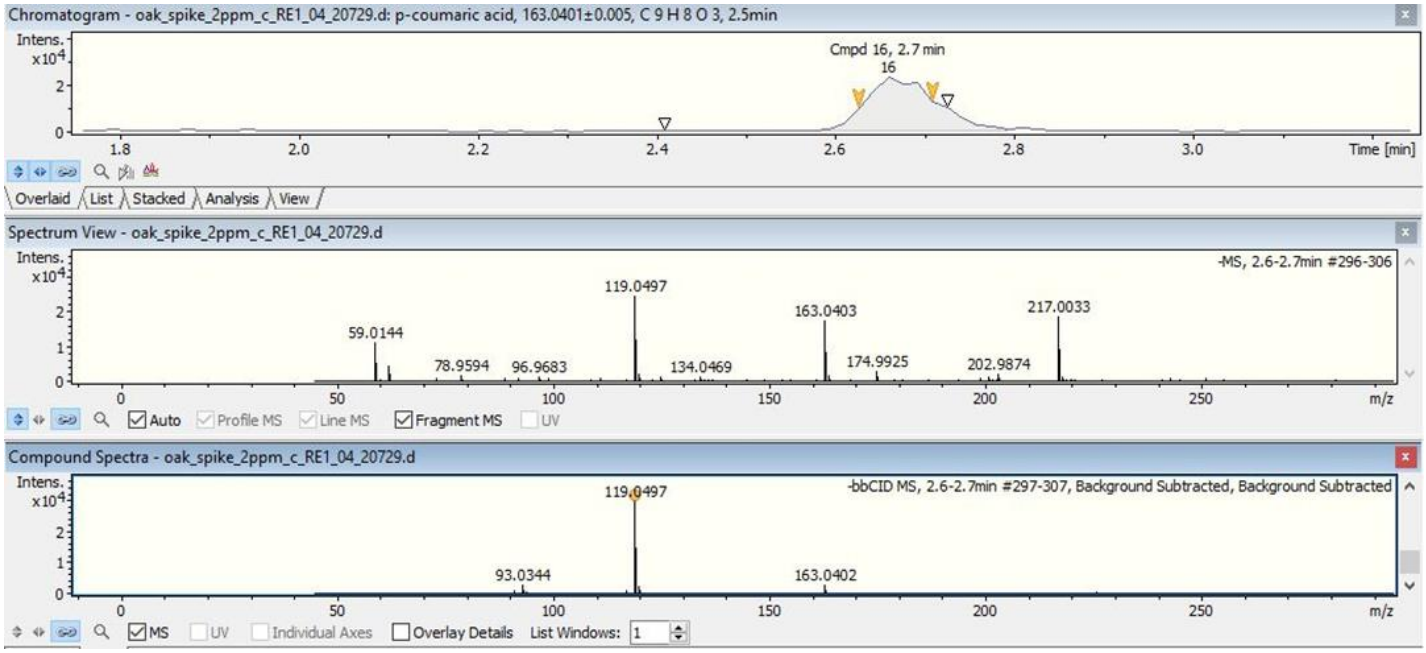


Figure 20. EIC, MS and MS/MS spectra of p-coumaric

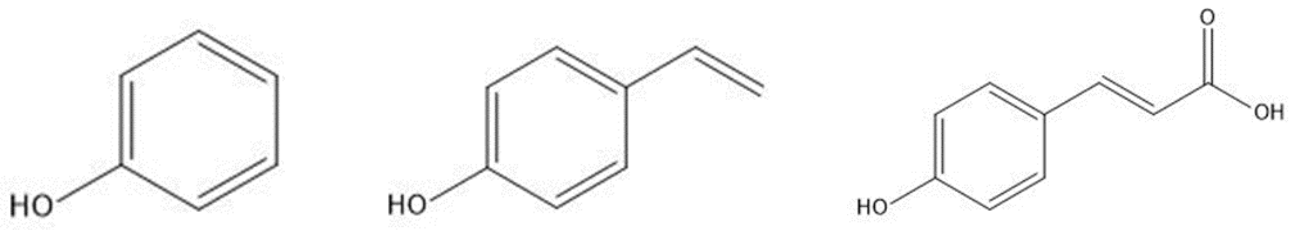


Figure 21. p-Coumaric acid molecule, alongside with the qualifier ions of the compound

5.5 Application of target screening approach to honey samples

5.5.1 Discrimination based on botanical origin

A total of 51 honey samples from nine different botanical origins have been analyzed in order to measure their phenolic content. Quantitative analysis is crucial to provide a comprehensive overview of the phenolic composition of unifloral honeys. The characterization of honey samples is extensively presented in section 4.2. In the next step, all samples were screened using the composed database in order to confirm the presence or absence of the target analytes. From a total of 22 polyphenols, 9 compounds were constantly determined namely apigenin, cinnamic acid, ferulic acid, luteolin, p-coumaric acid, quercetin, salicylic acid, taxifolin and vanillin. Table 9 summarizes the average concentration of each compound alongside with standard deviation (when more than 3 honey samples were measured). It has to be noticed that average concentration content of polyphenols has been calculated taking into consideration only the botanical origin of the samples.

Detected concentrations were between 0.038 ± 0.043 mg/Kg (quercetin in rape) and 5.0 mg/Kg (quercetin in fir). The majority of the determined compounds were below 0.5 mg/Kg. The results of table 9 are also depicted in figure 22 in order to visualize the variation of the determined concentration in the various unifloral honeys. To begin with fir honey showed a high amount of quercetin, about 5 mg/Kg, comparing to all other unifloral honeys. Moreover, heather honey showed a high content of cinnamic acid comparing to the other unifloral honeys. Although, the detected concentration of this compound was about 0.5 mg/Kg or lower in the other 9 honeys, cinnamic acid measured about 2.2 mg/Kg in heather honey. Acacia honey was the only unifloral honey with ferulic acid with concentration about 1 mg/Kg. In all other cases, ferulic acid was found lower than 0.2 mg/Kg. In this way, all the previously mentioned compounds may be used as potential authenticity markers. It has to be highlighted that a chemometric evaluation of the results

should be performed in order to obtain more reliable results. Regarding p-coumaric acid, it was found in concentration levels above 1 mg/Kg in many cases. In detail, 6 out of 10 unifloral honeys namely buckwheat, linden, rape, blossom, chestnut and thyme had a high p-coumaric acid content. Additionally, blossom, buckwheat and linden honey were determined with over 1 mg/Kg of salicylic acid. On the other hand, taxifolin was not detected in acacia, arbutus and linden honey.

Table 9. Concentration (mg/Kg) of phenolic compounds in various unifloral honeys

Honey matrix	**Acacia (n=5)**		**Arbutus (n=2)**	**Blossom (n=2)**	**Buckwheat (n=7)**		**Chestnut (n=2)**	**Fir (n=2)**	**Heather (n=9)**		**Linden (n=9)**		**Rape (n=7)**		**Thyme (n=3)**		
Compounds	avg	SD	*avg*	*avg*	avg	SD	*avg*	*avg*	*avg*	SD	*avg*	SD	*avg*	SD	*avg*	SD	*LOD*
Apigenin	<LOD	-	<LOD	<LOD	<LOD	-	<LOD	-	0.63	0.55	<LOD	-	<LOD	-	<LOD	-	0.15
Cinnamic acid	0.060	0.042	0.053	0.54	0.20	-	0.61	<LOD	2.3	2.2	0.081	0.027	0.067	0.043	0.046	-	0.029
Ferulic acid	0.95	0.74	0.049	0.14	0.18	0.14	0.18	<LOD	0.152	0.020	0.41	0.31	0.55	0.29	<LOD	-	0.083
Luteolin	<LOD	-	<LOD	0.18	<LOD	-	<LOD	-	<LOD	-	0.136	0.083	ND	-	<LOD	-	0.17
p-coumaric acid	0.25	0.18	<LOD	1.0	4.5	1.7	1.7	0.45	0.75	1.3	2.2	1.8	1.12	0.52	1.3	-	0.071
Quercetin	<LOD	-	0.19	0.049	0.20	0.18	0.054	5.0	0.20	0.10	0.26	0.25	0.038	0.043	0.152	0.049	0.025
Salicylic acid	0.48	0.34	0.16	1.2	1.61	0.39	ND	0.55	ND	-	1.3	1.1	0.70	0.31	0.53	0.52	0.023

Taxifolin	ND	-	ND	0.50	<LOD	-	0.15	0.61	0.068	0.064	ND	-	<LOD	-	0.190	0.096	0.034
Vanillin	0.22	0.12	<LOD	0.54	0.35	0.25	ND	0.11	0.31	0.26	0.22	0.11	0.33	0.28	0.16	0.14	0.042

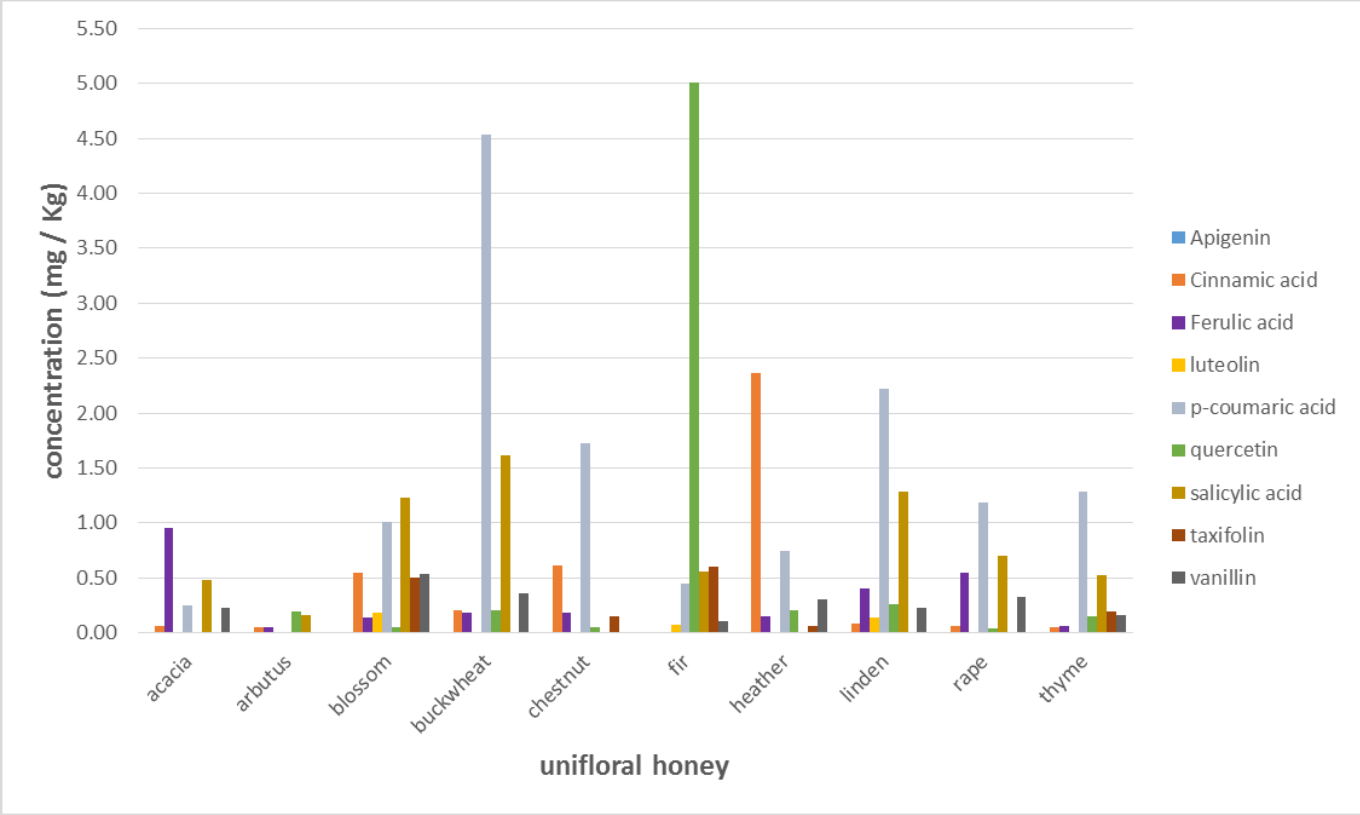


Figure 22. Phenolic profile of various unifloral honeys

This study comes to be added to the literature on polyphenols content in unifloral honey. Several studies have been performed both for the evaluation of phenolic acids and flavonoids content. Dimitrova et. al. [26] has focused on the profile of phenolic acids in various unifloral honeys. Concentration of the phenolic acids ranged between 0.3 and 15 mg/Kg. Among the determined compounds were ferulic, p-coumaric and salicylic acid in acacia, chestnut and heather honey. Phenolic content of acacia was in line with our study, while chestnut honey showed a lower phenolic content in our case. Furthermore, Can et. al. [124] had also reported the content of both phenolic acids and flavonoids. Four out of 18 compounds were determined both in this and our study, namely apigenin, ferulic acid, p-coumaric acid and quercetin. However, Can et. al. cited a higher value of polyphenols in unifloral honeys compared to our results. Apigenin, luteolin, p-coumaric acid and quercetin have been also found in acacia, buckwheat and linden

honey by Keckes et. al. [18]. Similarity in concentration values of these compounds was observed. In conclusion, it is essential to mention that similarities in concentration of specific compounds in unifloral honey may occur due to the dominant pollen feature of the honey type. However, full compatibility could not be determined. This means that honeys' phenolic composition do not depend only from floral but also from geographical origin.

5.5.2 Discrimination based on production type

The content of phenolic compounds in honey may be influenced by various factors, as it has already mentioned in section 2.5, such as botanical and geographical originin, climate and soil. According to our knowledge, there are not any scientific efforts focusing on the influence of production type on phenolic content. In this way, t-test was used in order to reveal if any statistically significant difference between the conventionally and organically produced unifloral honey exists. T-test can be used to determine if two sets of data are significantly different from each other. The t-test was performed using the function "TTEST" of Excel 2013. A two-tailed t-test with unequal variance performed between the average value of each compound. The number of available samples permits the application of the t-test only in two different unifloral honeys, namely buckwheat and linden. In figure 23 and 24 the concentration of each polyphenol is shown for both production types. Moreover, in table 10 the average concentration with standard deviation and p-value is presented. Phenolic compounds content fluctuates in organic and conventional honeys. Consequently, a phenolic compounds concentration pattern concerning production type cannot be depicted. According to buckwheat there is no significant difference in the concentration of polyphenols between conventional and organic honey. However, conventionally produced linden honey showed a significant higher concentration of vanillin comparing to organically produced one. To sum up, these results indicate that further investigation should be made in order to obtain a more reliable conclusion about the influence of production type on phenolic compounds content.

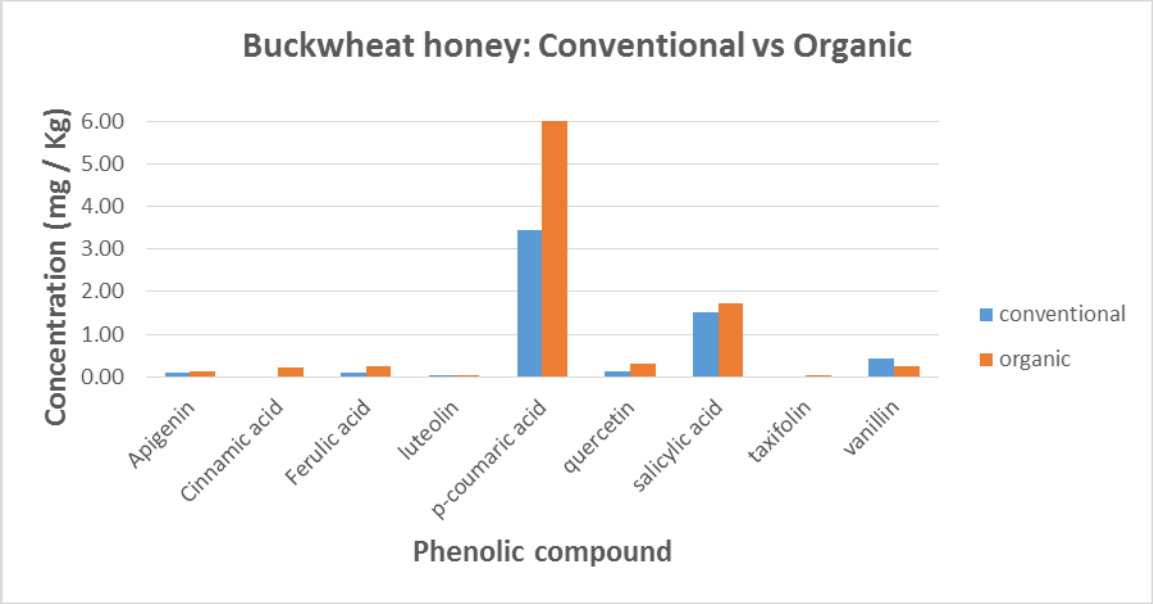


Figure 23. Phenolic compounds content in conventionally and organically produced buckwheat honey

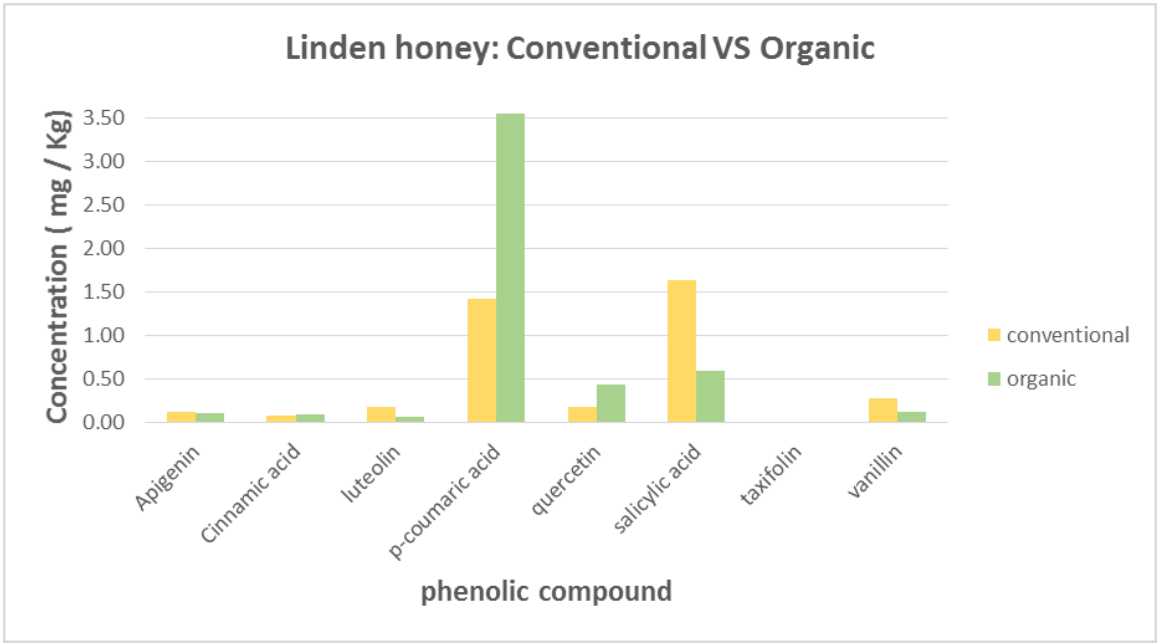


Figure 24. Phenolic compounds content in conventionally and organically produced linden honey

Table 10. Average concentration (mg/Kg), standard deviation and p-value of the determinded compounds in buckwheat and linden honey

	Buckwheat					Linden				
	Conventional (n=4)		Organic (n=3)			Conventional (n=6)		Organic (n=3)		
Compound	avg	SD	avg	SD	*p-value*	avg	SD	avg	SD	*p-value*
Apigenin	0.100	0.058	0.140	0.032	0.30	0.116	0.058	0.099	0.090	0.84
Cinnamic acid			0.203			0.072	0.045	0.087	0.019	0.71
Ferulic acid	0.100	0.048	0.24	0.16	0.27	0.23	0.12	0.75	0.30	0.09
luteolin	0.023	0.011	0.034	0.021	0.46	0.18	0.068	0.067	0.052	0.13
p-coumaric acid	3.4	1.6	6.0	2.3	0.19	1.41	0.49	3.5	2.5	0.28
quercetin	0.13	0.10	0.29	0.22	0.34	0.17	0.11	0.43	0.38	0.36
salicylic acid	1.52	0.83	1.73	0.42	0.68	1.6	1.2	0.60	0.60	0.13
taxifolin			0.024	0.010		-	-	-	-	-
vanillin	0.42	0.25	0.23	0.18	0.40	0.278	0.095	0.114	0.065	0.02

5.6 Suspect screening database

A suspect list of candidate phenolic compounds which have been already cited in previous studies was composed. The list included about 64 phenolic compounds cited in literature [15, 18, 27, 90, 114, 125-135]. After compiling the list, oak honey samples were scanned for the detection of these plausible polyphenols. The suspect peak list was acquired by using an automatic database search function Find Compounds-Chromatogram in Target Analysis (Bruker Daltonics) which created the base peak chromatograms for the masses of the list that attained the preselected threshold of intensity, excluding the isotopic peaks. In figure 25, the Target Analysis software environment is presented.

Some important criteria were applied in order to start the tentative identification procedure:

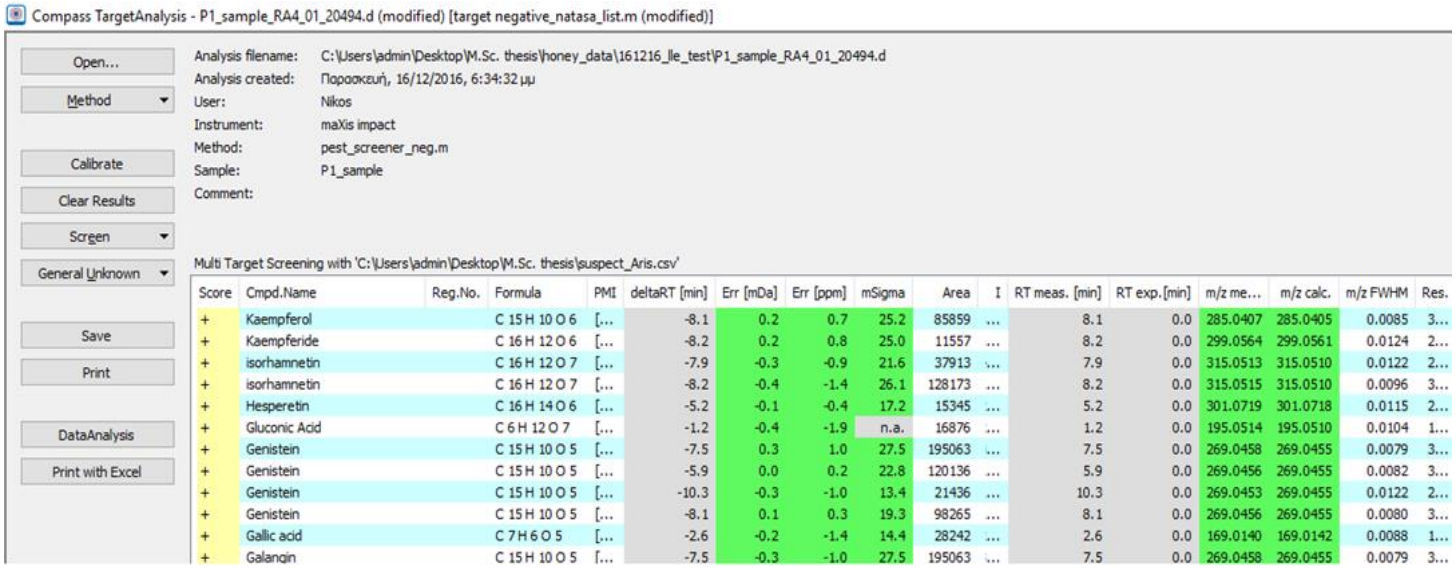
- absence of compounds in blank or zero-time samples
- mass accuracy threshold of 2 mDa and 5 ppm on the monoisotopic peaks
- maximum of 100 mSigma for the isotopic pattern fit, where mSigma-value represents the goodness of fit between the measured and the theoretical isotopic pattern (mass and ions ratios). However, mSigma was augmented in low intensity peaks.
- intensity threshold of 400 counts.

It is important to mention that the selected thresholds for fragments identification were more lenient due to their lower intensity:

- mass accuracy threshold of 10 ppm
- mSigma value the smaller the better (sometimes could not be calculated by the program due to low intensity of the isotopic ions)

5.7 Application

Oak honey samples were investigated in order to determine compounds from the suspect list. The screening procedure has been already described in the previous section. For the confirmation of the peaks that were in compliance with the screening criteria [33], spectral libraries like MassBank (www.massbank.eu) and an in-house retention time prediction program were utilized [121]. From the 64 suspect compounds, 3 different compounds were successfully identified and semi-quantified. In detail, the flavonoids chrysin, pinocembrin and pinobanskin. The detected compounds were semi-quantified using the calibration curve of apigenin, due to structural similarities of apigenin with these molecules. Table 11

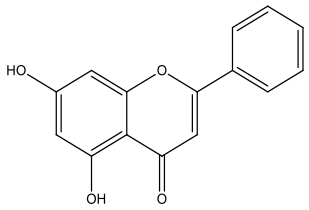
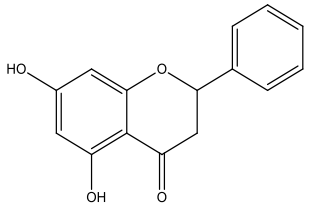
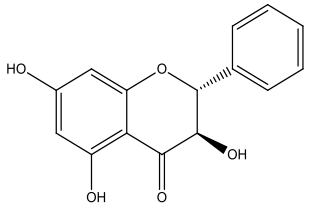


Score	Cmpd.Name	Reg.No.	Formula	PMI	deltaRT [min]	Err [mDa]	Err [ppm]	mSigma	Area	I	RT meas. [min]	RT exp.[min]	m/z me...	m/z calc.	m/z FWHM	Res.
+	Kaempferol		C 15 H 10 O 6	[...	-8.1	0.2	0.7	25.2	85859	...	8.1	0.0	285.0407	285.0405	0.0085	3...
+	Kaempferide		C 16 H 12 O 6	[...	-8.2	0.2	0.8	25.0	11557	...	8.2	0.0	299.0564	299.0561	0.0124	2...
+	isorhamnetin		C 16 H 12 O 7	[...	-7.9	-0.3	-0.9	21.6	37913	...	7.9	0.0	315.0513	315.0510	0.0122	2...
+	isorhamnetin		C 16 H 12 O 7	[...	-8.2	-0.4	-1.4	26.1	128173	...	8.2	0.0	315.0515	315.0510	0.0096	3...
+	Hesperetin		C 16 H 14 O 6	[...	-5.2	-0.1	-0.4	17.2	15345	...	5.2	0.0	301.0719	301.0718	0.0115	2...
+	Gluconic Acid		C 6 H 12 O 7	[...	-1.2	-0.4	-1.9	n.a.	16876	...	1.2	0.0	195.0514	195.0510	0.0104	1...
+	Genistein		C 15 H 10 O 5	[...	-7.5	0.3	1.0	27.5	195063	...	7.5	0.0	269.0458	269.0455	0.0079	3...
+	Genistein		C 15 H 10 O 5	[...	-5.9	0.0	0.2	22.8	120136	...	5.9	0.0	269.0456	269.0455	0.0082	3...
+	Genistein		C 15 H 10 O 5	[...	-10.3	-0.3	-1.0	13.4	21436	...	10.3	0.0	269.0453	269.0455	0.0122	2...
+	Genistein		C 15 H 10 O 5	[...	-8.1	0.1	0.3	19.3	98265	...	8.1	0.0	269.0456	269.0455	0.0080	3...
+	Gallic acid		C 7 H 6 O 5	[...	-2.6	-0.2	-1.4	14.4	28242	...	2.6	0.0	169.0140	169.0142	0.0088	1...
+	Galangin		C 15 H 10 O 5	[...	-7.5	-0.3	-1.0	27.5	195063	...	7.5	0.0	269.0458	269.0455	0.0079	3...

Figure 25. *Target Analysis (Bruker Daltonics) software*

summarizes the content of the three compounds alongside with the molecular formula and the chemical structure of each flavonoid. It has to be noticed that there are limited data concerning phenolic profile of oak honey indicating the importance of the present effort.

Table 11. Identified phenolic compounds in oak honey

oak honey			
compound	mg/Kg	molecular formula	chemical structure
chrysin	2.0	$C_{15}H_{10}O_4$	
pinocembrin	2.2	$C_{15}H_{12}O_4$	
pinobanskin	3.4	$C_{15}H_{12}O_5$	

The case of pinocembrin is chosen in order to show the steps that were followed for the identification of the suspect compounds. Firstly, the EIC of each suspected compound was extracted. Data Analysis 4.3 (Bruker Daltonics) was used and the environment of the software is presented in fig 26. The EIC, the MS and MS/MS spectrum were obtained. The fragments and the ratio between the fragments of MS/MS spectrum were compared with online spectrum from MassBank.eu. As it can be depicted by the fig 26 and 27 the pseudo molecular ion (in our study, m/z 255.0665 and in MassBank m/z 255.0667) and the qualifier ions (in our study 213.0560 and 151.0039 and in MassBank m/z 213.0554 and 151.0026) as well as the ratio between are matched in this two cases. Consequently, pinocembrin was identified and semi-quantified.

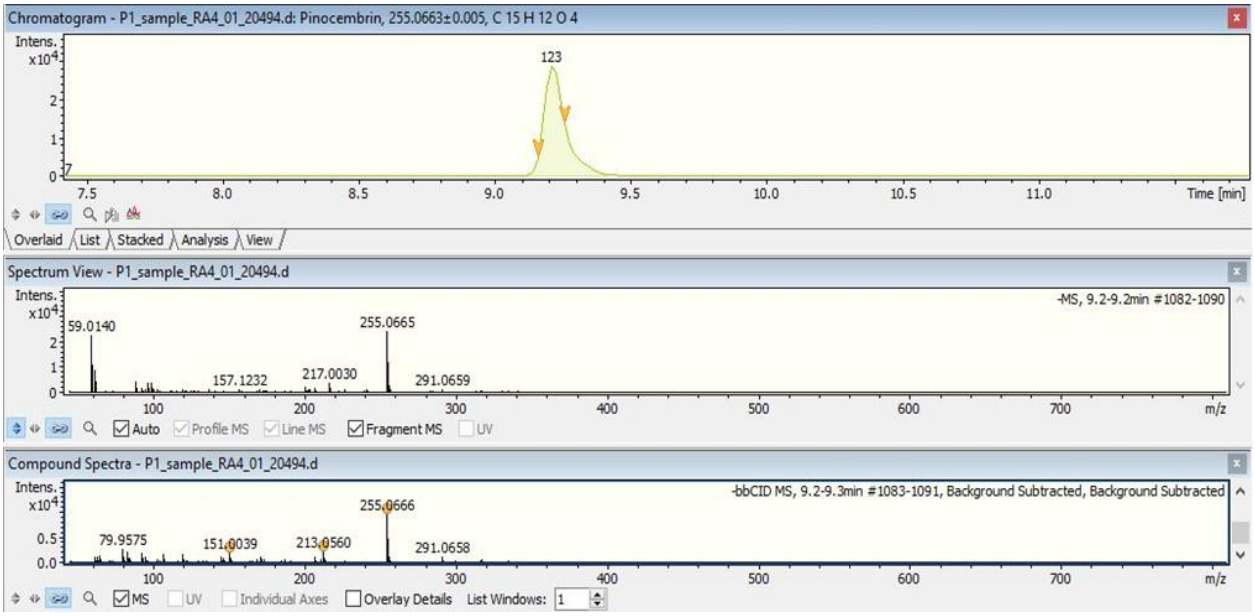


Figure 26. EIC, MS and MS/MS spectrum of pinocembrin

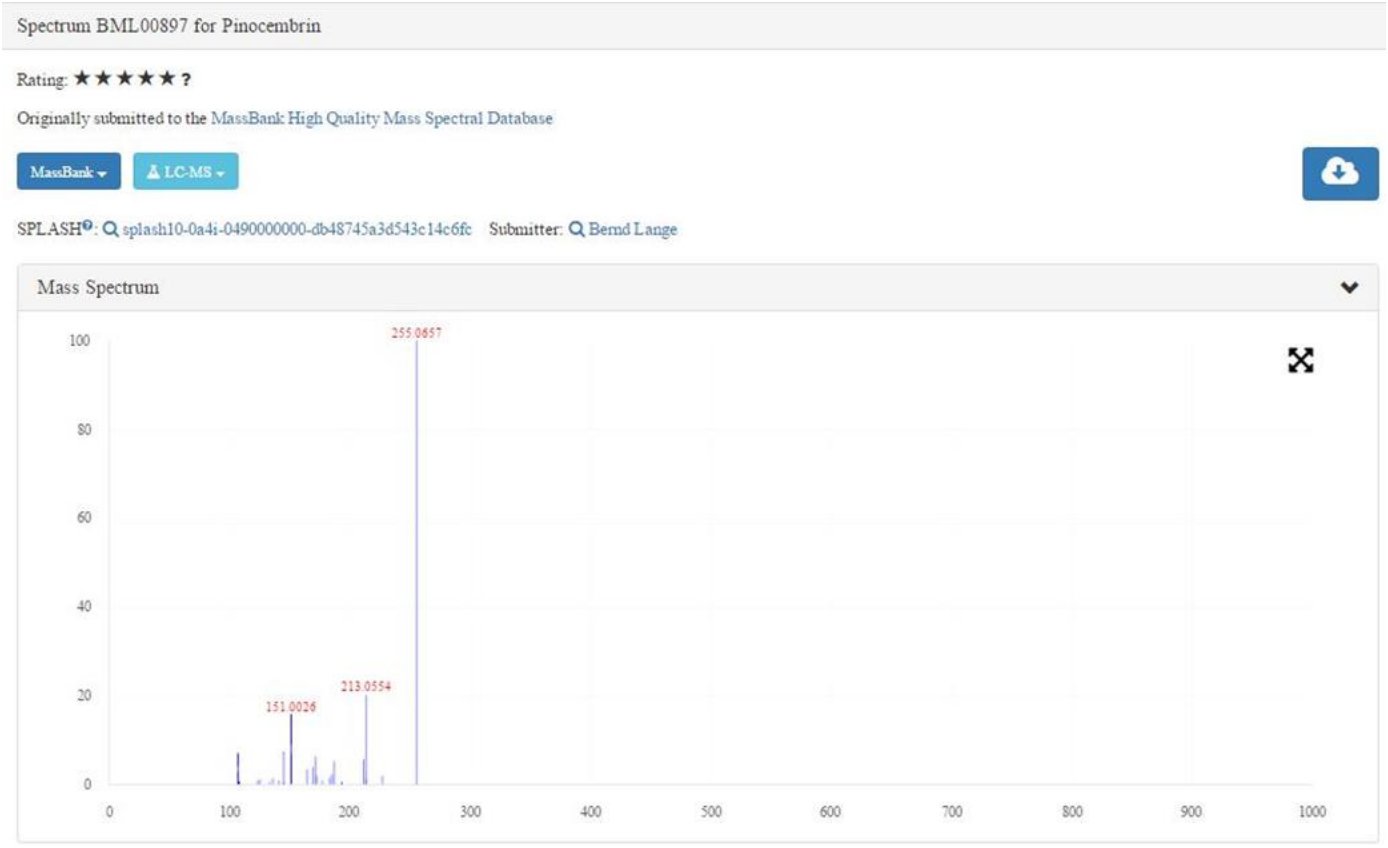


Figure 27. Online MS/MS spectrum of pinocembrin (www.massbank.eu)

CHAPTER 6
Conclusions

Honey authenticity is an issue which increasingly draws the attention of scientific community. Phenolic compounds, flavonoids and phenolic acids, are the most abundant antioxidant nutritive constituents and proved to be beneficial to human health. To this end, a method has been optimized and validated for the simultaneous determination of 22 phenolic compounds. EtAc was proven to be the most efficient extractant and this pretreatment may be used in metabolomic studies as it can extract a wide range of analytes. The developed method was tested and validated based on the Eurachem guidelines. All the quality characteristics of the method including linearity, LODs and LOQs, precision, matrix effect and trueness were more than acceptable. Consequently, the developed validated method was capable for the determination of phenolic compounds in honey.

Additionally, this master thesis evaluated the phenolic content of various unifloral honeys. The concentration of determined polyphenols ranged from 0.038 to 5.0 mg/Kg. The unifloral honeys with the greater phenolic content were buckwheat and fir honey, while acacia and arbutus honey contained low amount of these compounds.

Except the evaluation of the polyphenols content in each floral honey type, the presence or characteristic concentration of phenolic compounds linked to the botanical origin was investigated. In this way, fir honey featured a high quercetin concentration compared to the other matrices. Similarly, heather honey had a high amount of cinnamic acid while acacia honey was measured with an increased concentration of ferulic acid. Therefore, all the previously mentioned compounds may be used as potential authenticity markers. It has to be highlighted that chemometric tools should be used in order to reveal different phenolic compounds patterns which will result to more efficient and reliable discrimination.

The production type of honey was also investigated in order to find if organic production affects phenolic compounds content. According to our knowledge, this is the first effort concerning the impact of production type on phytochemicals. The number of available samples permitted the comparison of conventionally and organically produced honey only in the cases of buckwheat and linden honey. A two-tailed t-test with unequal variance performed between the average values of each compound. Organically produced linden honey showed a significant higher concentration of vanillin comparing to conventionally produced one. Nevertheless, phenolic content of buckwheat was not influenced by the production type. These results indicate that further investigation should be made in order understand if there is any influence of the production type on polyphenols content.

An in-house suspect list, containing phenolic compounds cited in previous studies, was composed. Oak honey samples were tested and three flavonoids were identified and semi-quantified. Namely, chrysin, pinocembrin and pinobanskin.

To conclude, the results obtained in the present work illustrate the importance of investigating polyphenols content in honey authenticity studies. It has to be highlighted that suspect and non-target screening should be performed for all the available samples. In this way, all the information obtained by the analysis will be utilized and authenticity markers alongside with discrimination patterns will be revealed.

ABBREVIATIONS AND ACRONYMS

Table 12: Abbreviations and acronyms

5-HMF	5-hydroxymethylfurfural
ACN	Acetonitrile
AAS	Atomic Absorption Spectroscopy
AD	Anno Domin
AOC	Antioxidant capacity
AUA	Agricultural University of Athens
avg	Average Value
bbCID	broadband Collision-Induced Dissociation
CAP	Common Agricultural Policy
CRM	Certified Reference Materials
DFAs	Difructose Anhydrides
DN	Diastase Number
EIC	Extracted Ion Chromatogram
ESI	ElectroSpray Ionization
EtAC	Ethyl Acetate
FIA	Flow Injection Analysis
FT-ICR	Fourier Transform-Ion Cyclotron Resonance
GC	Gas Chromatography
GC-MS	Gas Chromatography coupled to Mass Spectrometry
GIs	Geographical Indications

HFCS	High Fructose Corn Syrup
HPAEC	High Performance Anion Exchange Chromatography
HPLC	High-Performance Liquid Chromatography
HR-MS	High Resolution Mass Spectrometry
HS	HeadSpace
IC	Ion Chromatography
ICP-MS	Inductively Coupled Plasma – Mass Spectrometry
ICP-OES	Inductively Coupled Plasma – Optical Emission Spectroscopy
INAO	Institut National des Appellations d'Origine
LC	Liquid Chromatography
LC-HRMS	Liquid Chromatography coupled to High Resolution Mass Spectrometry
LC-MS/MS	Liquid Chromatography tandem Mass Spectrometry
LC-QqQ MS/MS	Liquid Chromatography coupled with triple-Quadrupole Mass Spectrometry
LC-QTOF-MS	Liquid Chromatography–Quadrupole-Time-Of-Flight-Mass Spectrometry
LDL	Low-Density Lipoprotein
LLE	Liquid-Liquid Extraction
LODs	Limits Of Detection
LOQs	Limits Of Quantification
MALDI	Matrix Assisted Laser Desorption Ionization

ME	Matrix Effect
MeOH	Methanol
MF	Matrix Factor
MS	Mass Spectrometry
MWCNTs	Multiwalled Carbon Nanotubes
ND	NonDetected
NIR	Near Infrared Spectroscopy
NKUA	National and Kapodistrian University of Athens
OAs	Organic Acids
ODS	OctaDecylSilane
PCA	principal component analysis
PDO	Protected Designation of Origin
PGI	Protected Geographical Indication
pH	potential of Hydrogen
PLS	Partial Least Squares
QqQ	TripleQuadrupole
QToF	quadrapole time of flight
RDI	Refractive Detector Index
REEs	Rare Earth Elements
RP	Reversed Phase
RPLC-QTOF-MS	Reversed Phase Liquid Chromatography-Quadrupole-Time-Of-Flight Mass Spectrometry

RSD	Relative Standard Deviation
SCIRA	Stable Carbon Isotopic Ratio Analysis
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SPE	Solid-Phase Extraction
SPME	Solid-Phase Micro-Extraction
SRM	Selected Reaction Monitoring
TOF	Time-Of-Flight
t_R	retention time
TSG	Traditional Specialities Guaranteed
UHPLC	UltraHigh-Performance Liquid Chromatography
USDA	United States Department of Agriculture
USE	Ultra-Sound Extraction
UV	Ultra Violet
UV-Vis	Ultra Violet - Visible
VOCs	Volatile Organic Compounds

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